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The major cause of cancer deaths can be attributed to metastasis. Our goal is to identify metastasis-controlling genes for human breast cancer. This research is based upon our finding that microcell-mediated transfer of chromosome 11 into MDA-MB-435 results in nearly complete suppression of metastasis without suppressing tumorigenicity.

The key findings during this reporting period were: (1) *Kai-1* mRNA expression levels correlate with metastatic potential of a panel of human breast carcinoma cell lines; (2) transfection of *Kai-1* partially suppresses metastasis of MDA-MB-435; (3) increased expression of PKCδ correlates with metastatic propensity; and (4) transfection with MEK1 transforms NIH3T3 cells and makes them metastatic.

Preliminary results include: (1) Using differential display, we also identified six novel differentially expressed genes in neo11/435 hybrids compared to controls. Obtaining full-length cDNAs and characterization are underway. We have also begun selection (3X) for enhanced lung colonization potential of MDA-MB-435 variants.

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PI - Signature 13 July 98 Date

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INTRODUCTION

Experimental Context

The most dangerous attribute of cancer cells is metastasis. Our objective is to determine the molecular mechanisms responsible for controlling breast cancer spread. The timing and location of nonrandom karyotypic abnormalities has provided clues regarding the genes involved in breast carcinoma progression. In breast cancer, structural changes frequently involve chromosomes 1, 8, 11, 13, 16 and 17. Chromosomes 8, 13 and 17 changes generally occur early in progression; whereas, deletions and rearrangements of chromosomes 1, 6, 11 and 16 often occur later [(1)]. As a corollary, one would hypothesize that genes relevant to breast cancer progression toward metastasis are encoded on the latter chromosomes. To test this hypothesis, we introduced an intact, normal human chromosome 11 into the metastatic human breast carcinoma cell line, MDA-MB-435 using microcell-mediated chromosome transfer (MMCT). We showed that metastasis was suppressed by 95%, but tumorigenicity was unaffected [(2)]. This finding suggested the presence of at least one human breast carcinoma metastasis-suppressor gene on chromosome 11. ***Please note***: We define a metastasis-suppressor gene as blocking tumor spread. A *tumor* suppressor gene would suppress tumor growth and, by inference, metastasis as well.

The goal of DAMD-17-1-96-6152 is to map (and hopefully clone) the gene(s) on chromosome 11 responsible for metastasis suppression. In addition, we want to test whether similar metastasis suppression occurs if chromosome 11 is introduced into other metastatic human breast carcinoma cell lines. These technical objectives fall within the ultimate goal of understanding the mechanisms underlying breast cancer metastasis.

Background

Metastasis results from accumulated genetic changes from which a subset of late-stage cancer cells evolve that are no longer confined to their tissue of origin for growth. In order to successfully colonize a distant organ, metastatic cells must survive transport through the body, interact with a variety of host cells and successfully penetrate numerous barriers. If a cell cannot complete every step, it is nonmetastatic. The multistep metastatic cascade involves numerous genes [(1;3-6)]. Two classes of metastasis-associated genes have been identified — (i) genes that drive metastasis formation, and (ii) genes that inhibit metastasis. However, the identities of most of these genes remain unknown. Correspondingly, it is not known how these genes are regulated in normal and/or cancer cells. Nonetheless, it is well recognized that the probability for long-term survival is extremely low if metastases develop.

In addition to the findings mentioned above, we have made four observations relevant to the genetics of human breast cancer metastasis. (1) Transfection of KiSS-1, a novel metastasis-suppressor gene discovered in our laboratory [(7)], suppresses metastasis by at least 50% [(8)]. (2) Expression prostate cancer metastasis-suppressor gene, KAI-1 [(9)], correlates with breast tumor aggressiveness [(2;10)]. When KAI-1 cDNA was transfected into MDA-MB-435 cells, metastatic potential decreased significantly [(11)]. (3) Expression of the delta (δ) isoform of protein kinase C correlates directly with metastatic potential of related rat mammary carcinoma cells [(12)]. (4) Mutant forms of MEK1 (**M**ap Kinase/**E**rk **K**inase) when transfected into NIH3T3 cells confer not only tumorigenicity, but also metastatic potential [(13;14)].

BODY
-EXPERIMENTAL METHODS-

Rationale (Global)

Positional cloning has been used to identify a number of tumor-suppressor genes (e.g., WT1, Rb, FHIT) and genes for mutations that predispose cancer susceptibility (e.g, NF1, APC) (reviewed in [(15)]). As mapping nears completion, detection of mutations among cancer families confirms a particular gene's

role as a tumor suppressor. Since mutations are relatively rare, equally strong evidence for a role in cancer etiology is required. Thus, positional cloning is reasonable if strong, well-characterized pedigrees are available. However, determining roles for genes in sporadic tumors or progression-associated genes (e.g., metastasis-controlling) is difficult because of tumor heterogeneity, genetic instability and the huge number of experiments necessary to prove causality. This is further complicated for multigenic phenotypes, like metastasis. Simply, the statistical likelihood for identifying a specific gene over the immense background of genetic instability typical of late-stage tumors is difficult. Thus, alternative approaches are required.

MMCT (microcell-mediated chromosome transfer) has provided functional evidence for tumor suppressor genes when other approaches have failed [16;17]. The functional data have provided the necessary information for successful mapping of the genes responsible [18-34]. As an intermediate, some have utilized a modification of MMCT in which the donor chromosome has been irradiated to produce deletions [23;28;35-38]. This modification is based upon a loss of function (i.e., failure to suppress) associated with the deletion.

The strategies we proposed for identifying metastasis-controlling genes in human breast cancer were based upon those listed above as well as those we used to identify novel metastasis-suppressor genes in human melanoma [7;39-42]. Basically, two concurrent approaches were outlined. First, progressively smaller fragments of neo-tagged human chromosome 11 were to be introduced into MDA-MB-435 by MMCT. By evaluating regions of overlap for chromosomal fragments present/absent in suppressed/non-suppressed hybrids, the location of the putative metastasis-suppressor gene(s) would be defined. The second approach was to use differential display [43;44] and subtractive hybridization [45;46]. Once candidate genes were identified, transfections and testing for metastasis in appropriate animal models would confirm that a *bona fide* metastasis-suppressor gene had been cloned.

The second major objective of DAMD-17-1-96-6152 was to demonstrate the introduction of chromosome 11 into another metastatic human breast carcinoma also suppresses metastasis.

This progress report will be organized in the following manner. Each section summarizes results from related series of experiments. The relationship of those experiments to a particular Specific Aim is noted. Only new data, collected since submission of the FY96-97 progress report, is included.

Section 1: **Welch, D.R. and Wei, L.L. (1998) Molecular control of breast cancer progression and metastasis. *Endocrine Related Cancers* (In press)**

Summary of major findings: This was an invited paper in which I was asked to review briefly the literature about metastasis-controlling genes in human breast cancer, particularly genes which are hormonally regulated. However, upon reviewing the literature, I identified >8000 papers which claimed to present data showing association between metastasis and particular genes. This necessitated that the breadth of the review be expanded in order to review the role of genes in breast cancer at "all" stages of progression. Basically, most papers speculated on a role of genes in invasion, progression and metastasis but presented no data to support such claims. Additionally, the problems associated with ill-defined model systems (i.e., what kind of breast cancer is being studied?) was addressed.

While not directly addressing a specific aim from the original proposal, this review was extremely useful for formulating and modifying my thinking about breast cancer genetics. During the writing process, I had to address many issues related to breast cancer metastasis research and organize them. The critical review also helped us focus on key issues which need to be addressed in order to accomplish the aims set forth for this program.

Section 2: **Introduction of chromosome 11 into MDA-MB-231 [Unpublished]**

Rationale

The objective of these experiments is to determine whether introduction of chromosome 11 into another human breast carcinoma causes metastasis suppression. To date, only three metastasis-suppressor genes have been shown to suppress metastasis of human breast cancer in an in vivo model — Nm23-H1, KiSS-1 and KAI-1. And regarding chromosomal location of metastasis-controlling genes, only one publication exists [2]. All of these papers have used only once cell line, MDA-MB-435, since it is the only reproducibly metastatic breast carcinoma cell line. None of the papers address whether these genes or chromosomes are functioning in other types of breast carcinoma (i.e., not infiltrating ductal).

Summary of major findings

Although most human breast carcinoma cell lines were derived from metastatic lesions or pleural effusions, most do not metastasize in experimental animal models [47]. The MDA-MB-231 has been reported in the literature to be metastatic [48;49]. So, we obtained these cells from Drs. Garth Nicolson (Institute for Molecular Medicine, Irvine, CA), Robert Gillies (University of Arizona Cancer Center) and David Rose (American Health Foundation). Before performing MMCT, we wanted to verify metastatic potential. Injection of cells (up to 1 x 10^7) into the mammary fat pads of athymic nude mice produced the following results.

Nicolson variant — no tumors (We later learned that these cells had previously been infected with *Mycoplasma* and the infection had been eliminated. Apparently, they underwent a selection and none of the remaining cells were metastatic. Dr. Nicolson confirmed our findings.)

Gillies variant — only 20-30% of animals formed tumors, only 5% (1/20) of mice had a metastasis to the draining axillary lymph node. None had metastases in viscera. After discussions with Dr. Gillies, we cannot explain the differences in our results unless SCID mice are necessary to observe metastasis from his variant.

Rose variant — *Mycoplasma* contaminated (We notified Dr. Rose and have discarded to culture. He has informed me (6/24/98) that the *Mycoplasma* has been eliminated and that the cells have been injected into mice. If they grow and metastasize, he will re-send another culture to us.)

Recommendations for follow-up experiments based upon these results

Evaluate additional breast cancer cell lines to assess metastatic potential

1. We have also been in contact with Dr. Janet Price (U.T.-M.D. Anderson Cancer Center, Houston, TX) who has recently isolated a "highly" metastatic variant of MDA-MB-231, designated MDA-MB-231/S1. We recently received this line (May 1998) and are expanding the culture for freezing and expect to do injections on July 15, 1998 (when space is available).

2. We have tested SUM breast carcinoma cell lines isolated by Dr. Steven Ethier (University of Michigan) who has an USAMRDC Infrastructure Grant. SUM149 cells developed ipsilateral axillary lymph node metastases in 3/16 mice following injection of tumor cells into the mammary fat pad. Given that the metastases could be direct extension of the primary tumor, we decided not to test this line further. (Note: Based upon experience and relatively low incidence of lymph node metastases, we believe that it is likely that the lymph nodes were involved because the tumor grew directly to the node and "engulfed" it.) Dr. Ethier sent us four additional SUM cell lines (SUM185, SUM 190, SUM229, SUM1315M02) in June 1998 which we agreed to test *in vivo*. These lines were chosen because they have *in vitro* properties that are "more aggressive" than most of the others he is developing. The cells have been thawed and are being expanded for injection in mid- to late-July (due to slow *in vitro* growth rate).

3. We have contacted several other investigators and have standing requests for highly aggressive human breast carcinoma cell lines; however, no one has yet provided any.

4. Since MDA-MB-435 is metastatic and heterogeneous, we decided that isolation of single cell

clones would be helpful. We rationalized that comparison of karyotypes and sequence-tagged sites for clones of different metastatic potentials might provide insights into the region(s) involved in metastasis. Limiting dilution cloning was used to isolate single cell clones from the parental MDA-MB-435 population. Similar experiments were performed with pSV2neo-transfected and pcDNA3neo-transfected MDA-MB-435 cells. The neo-transfectant clones were isolated in order to control for MMCT experiments and as part of transfection experiments (See below).

It has been frustrating that the metastatic potential of single cell clones has been so variable (inter-experimental). Most clones were tested at least twice during the past year. In general, trends were the same; however, there is still more variability than desired. Use of the mixed population, while not ideal, still represents the best option at this point. Another unexplained frustration is that almost all transfectants with empty vector have generally lower metastatic potential than the parental mean. Thus far, we have not obtained a more metastatic clone. Since this is a passive experiment (i.e., clones are isolated as a regular part of other experiments), we will continue. However, our expectations are rather low.

5. We began to select, in a manner analogous to Fidler[50], increasingly metastatic subpopulations from MDA-MB-435. The rationale is that sequential selection of lung colonies will enrich for highly metastatic variants. Three rounds of *in vivo* selection have taken place and the results are shown in **TABLE 1**. In general, we see an increase in the number of lung metastases per mouse with the selections. The variability observed is consistent with the types of numbers seen in other selection schemes (e.g., B16 melanoma). Although nine (9) metastases per mouse was lower than our historical cumulative average (16 lung metastases per mouse), we have nearly doubled the metastatic efficiency with three only selections. In addition, although we are not quantifying on the basis of size, most lung metastases appear larger and are more readily detected. We will continue with this selection since it has the potential to be useful for future experiments and because it requires relatively little effort.

Table 1: Selection of lung colonizing variants of MDA-MB-435

Cell Line	No. Selections	Incidence of lung metastases	No. lung metastases per mouse	Incidence of RLN metastases
MDA-MB-435	0	9/9	9 ± 3	8/9
435-Ln1	1	15/15	71 ± 20	10/15
435-Ln2	2	7/7	29 ± 10	5/7
435-Ln3	3	17/17	24 ± 7	17/17

MDA-MB-435 cells (1 x 10^6) were injected into the mammary fat pads of athymic mice. When local tumors reached 1.3-1.5 cm mean tumor diameter, they were removed. Four weeks later, mice were killed and examined for presence of metastases. To obtain cell lines, large lung metastases were removed aseptically, rinsed 10-20 times in sterile saline, and minced with scalpels using a cross-cut motion. Tissue pieces were placed into culture medium and grown under standard conditions. Before repeating *in vivo* studies, cells were verified to be free from *Mycoplasma* contamination. Incidence of regional lymph node (RLN) metastases — typically ipsilateral and contralateral axillary nodes — was evaluated as well.

Section 3: **Evaluation of KAI-1 as a metastasis-suppressor gene in human breast cancer**

Rationale: neo11/MDA-MB-435 hybrids expressed more KAI-1 mRNA than parental MDA-MB-435 cells. Since KAI-1 is encoded on 11p and since it has demonstrable metastasis-suppressor ability, it became a prime candidate in our studies. Two experimental strategies were undertaken to assess the rele-

vance of this finding. These experiments constituted the initial experiments described in Specific Aim 4.

Summary: The first approach was to compare mRNA expression in a panel of human breast cells representing varying degrees of aggressiveness. We did this study in collaboration with Dr. Lisa Wei who was, at that time, still in Hershey. We used mRNA initially because antibodies were not available at that time. Briefly, KAI-1 expression inversely correlated with tumor aggressiveness [10].

The second approach more directly tested the hypothesis. We initiated this study, but since reagents were not commercially available and it was highly desirable to obtain the results quickly, we worked closely with Drs. Barrett and Weissman. All of the *in vivo* assays were done by us. In short, KAI-1 transfectants were significantly suppressed for metastasis; however, the level of suppression was not as impressive as by chromosome 11 itself. Eventually, we were able to obtain an antibody that recognizes KAI-1 protein. Western blotting showed that the interpretation is complicated by altered glycosylation [11]. Although the data are consistent with the notion that KAI-1 is a metastasis-suppressor, we are dubious. This is based partially upon intuition and reports of unpublished data that Kai-1 is not suppressive in human prostatic carcinoma. I have reviewed eight manuscripts during the past six months and most show excellent correlations with metastatic potential, but few showed functional evidence of metastasis suppression.

Recommendations for follow-up experiments based upon these results: We have opted to forego further studies of Kai-1.

Section 4: **MMCT of pieces of chromosome 11 into MDA-MB-435 [Unpublished]**

Rationale

This results reported in this section are based upon the strategy proposed for Specific Aim 1 in the original proposal. The objective is to map the gene(s) on chromosome 11 responsible for metastasis suppression to within 5 Mb. Then we want to determine how the gene(s) work. The strategy was to introduce progressively smaller pieces of chromosome 11 or to introduce fragments of chromosome 11 with overlapping deletions.

Our primary strategy was to prepare chromosome 11 microcell donors that have deletions as a result of radiation damage [35;51-53]. Deletion mutants would then be introduced by MMCT into MDA-MB-435 followed by assessment of metastasis in athymic nude mice. With this approach, random deletions need not be mapped beforehand. They can be mapped following fusion based upon predetermined polymorphisms spanning chromosome 11. If the metastasis-suppressor gene is retained, functional complementation of the defect will be repaired and the cells will be nonmetastatic. If the gene has been deleted, suppression will not occur. Metastatic hybrids would then be evaluated for portions of the chromosome 11 retained. Position of the metastasis-suppressor gene can be inferred by the smallest region of shared deletion. This has most recently been used to clone tumor or growth suppressor genes for prostate cancer [37], breast cancer [38;54], glioma, and head and neck squamous cell carcinomas [26]. We recently used this approach and verified that the PTEN/MMAC1 phosphatase gene functions as a tumor suppressor in some human melanoma cell lines [33].

The second approach is to utilize MMCT donors with previously defined fragments of chromosome 11 [52;53;55]. The advantage of this approach is that fully-defined DNA is introduced into the cells. While aesthetically pleasing, the time required to fully characterize the donor chromosome fragment can take months to years.

Initially, the second approach was only to be a contingency because characterization of chromosome donors is highly labor intensive. However, we are taking advantage of a collaboration with Drs. Jane Fountain (University of Southern California), Tracy Lugo (formerly of the University of California – Riverside and now at NIH) and Gavin Robertson (Ludwig Cancer Center, University of California-San

Diego) where our objective is to map melanoma tumor suppressor genes on chromosome 11 [53].

They had prepared a panel of karyotypically defined chromosome 11 fragments. Therefore, we began making hybrids using fragments as donors. For unexplained reasons, transfer has not proceeded efficiently. Some of this is due to personnel turnover and training time, but all three labs have had difficulty recently. Nonetheless, some hybrids have been prepared and results are listed in **Table 2**.

Summary of findings and recommendations for follow-up experiments

Progress on Specific Aim 1 has been frustratingly slow. Part of the problem was technical — Sigma could not fill orders for the lectin used for MMCT. The high-activity lectin was back-ordered for almost 5 months. We tried the lower activity lectin, but were not successful for any of the hybridizations done with either breast or melanoma cells. Once we obtained good lectin, we got colonies but basically the hybridizations were still not productive. Therefore, we have contemplated other approaches.

After several discussions, Dr. Fountain and I have concluded that it might be worthwhile to take advantage of the large body of published positional cloning (loss of heterozygosity) data from clinical samples to map hot spots in breast cancer. This would to obtain large-insert vector forms (P1, PAC, BAC, YAC ...) which could then be retrofitted with selectable markers. Vectors are now available to retrofit P1, BAC or PAC clones [56]. Based upon their relatively large average insert size of BAC/PAC/P1 (100-200 kb), it has become feasible to individually transfect P1 or PAC clones into breast carcinoma cells. Even with 3 chromosomal regions of 1 Mb each (total 3 Mb), the maximum number of transfectants would be 300. Although this number is not trivial, we estimate that the quantity of work for retrofitting and transfection would be estimated in months rather than years for the chromosome pieces. Given that the efficiency of transformation with these vectors is more efficient than MMCT, the probability for success would be higher. In addition, the P1, BAC and PAC clones have relatively low recombination frequencies (unlike YAC and even chromosome fragments), making their use "safer" for introduction into mammalian cells. Since the chromosome pieces are generated using radiation, we always run the risk of false negative results because an (in)active point mutant has been introduced. Therefore, Dr. Fountain and I have requested the retrofit vectors and expect to begin this approach as a pilot series of experiments in July. Since we are both looking for genes on chromosome 11, progress should be easily accessible in the short

Table2: Status of chromosome 11 MMCT into MDA-MB-435

Chromosome 11 donor	No. attempts	Status	Results and Interpretation
4.S2	5	no colonies	
E53	5	no colonies	
53	3	no colonies	
1.S2	7	grew for several passages then died	Two colonies frozen, PCR of early colonies showed that they retained the chromosomal fragment. We speculate that the chromosome fragment may contain a senescence moiety.
7	4	4 colonies	One colony grew very slowly *in vitro* (i.e., doubling time approximately 2 weeks!). Upon injection into mice, a 2 cm tumor was present within 30 days. This was faster than the MDA-MB-435 parent. When the tumor was re-established into cell culture, it grew as slowly as before. The mouse had lung metastases.

term. Once the retrofit vectors are prepared, they will be transfected into MDA-MB-435 (or other lines) followed by assessment of metastatic potential.

Section 5: **Use of differential display to identify metastasis-suppressor genes on chromosome 11 [Unpublished]**

Rationale: In addition to the strategy of mapping the gene(s) on chromosome 11 using MMCT, we also proposed use of differential display [43;44;57] and subtractive hybridization approaches to identify genes differentially expressed in the neo11/MDA-MB-435 hybrids. The latter paralleled the approach we used to identify the human melanoma metastasis-suppressor gene KiSS-1 [7;41]. These strategies were outlined as Specific Aim 3.

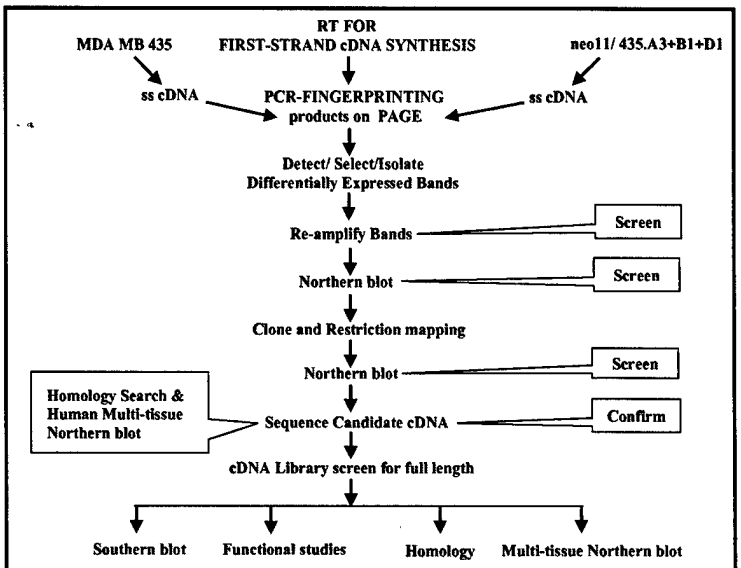

Figure 1: Experimental design for identifying metastasis-suppressor genes on human chromosome 11.

A flow chart showing the basic experimental design for differential display is shown in **Figure 1**. Briefly, differential display is repeated in independent reactions in order to minimize artifactual amplification. Bands are excised and PCR is performed on those bands with the same primers. Failure to re-amplify excluded that band from further consideration. The cDNA is then used to probe a "screening" northern blot which has the most metastatic and least metastatic variants. Appropriate expression (i.e., cDNAs expressed exclusively in nonmetastatic cells or >5-fold greater expression in the nonmetastatic cells) is used as a criterion for continued interest. The candidates are then tested in a more extensive panel of cells and continued "appropriate" differential expression is necessary for subsequent molecular characterization. While we originally planned to evaluate candidates whose expression was at least 10-fold greater in neo11/MDA-MB-435 clones, this criterion turned out to be too stringent. We believe this is due to the heterogeneity within the parental population.

cDNA libraries were constructed from neo11/MDA-MB-435.B1 (approximately 10^6-10^7 λZAP II plaques containing average insert size of 1.0-1.5 kb). Heteroduplexes prepared from first strand neo11/MDA-MB-435.B1 cDNA and biotinylated MDA-MB-435.1 mRNA were reacted with streptavidin before extraction using phenol : chloroform. Unbound single strand cDNA constituted the subtracted library and was used to probe Northern blots. No consistent differences were identified using this approach. Therefore, differential display was used. In the previous progress report, we reported identification of 11 candidate cDNAs. However, none of the results were consistent or reproducible.

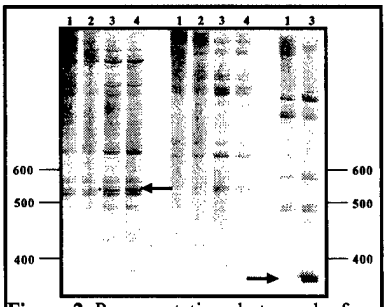

Figure 2. Representative photograph of a differential display gel. Lanes 1 & 2 and 3 &4 are different concentrations of the same template. Lanes 1 & 2 are from MDA-MB-435 and Lanes 3 & 4 are from equimolar mixtures from neo11/MDA-MB-435 clones A3, B1 and D1.

Therefore, a second differential display was done using the more stringent criteria and adaptations depicted in **Figure 1**. Primarily, a mixture of mRNAs from neo11/MDA-MB-435 clones was used, rather than mRNA from a single clone. A representative gel is shown in **Figure 2**. Eighteen (18) of the differentially expressed bands were

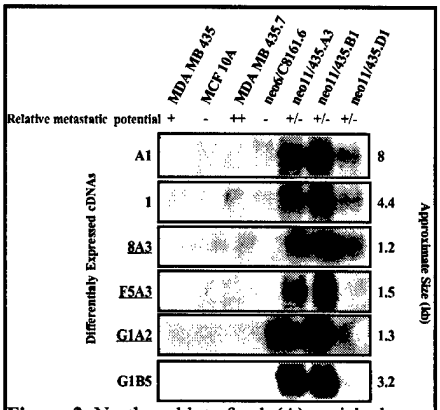

Figure 3: Northern blot of poly(A)-enriched RNA (2.5 µg) using candidate partial cDNAs identified by differential display. Note: neo11/MDA-MB-435 clones show significantly higher expression than parental MDA-MB-435, MCF10A (near normal breast), and a subclone isolated from MDA-MB-435, clone 7. A negative control neo6/C8161.6 was included also. Equal loading was confirmed by GAPDH, but neo11/MDA-MB-435.D1 was under-loaded compared to other lanes.

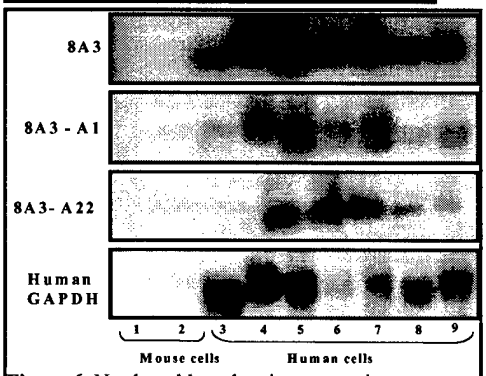

Figure 6: Northern blots showing expression pattern for 8A3 and cDNA clones isolated from a human placental cDNA library. Lanes 1 & 2 are mouse cells: A9 (cell from which chromosome 11 donor was obtained) and the chromosome 11 donor cell line. Lanes 3-9 are human cell lines. Lane 3, C8161.8 (human melanoma); Lane 4, MDA-MB-435; Lane 5, neo11/MDA-MB-435.D1; Lane 6, neo11/MDA-MB-435.B1; Lane 7, neo11/MDA-MB-435.A3; Lane 8, MDA-MB-231; and Lane 9, MDA-MB-435.

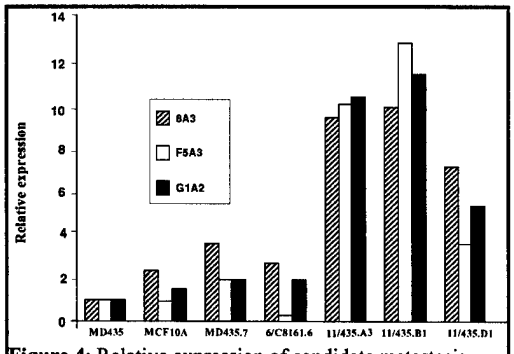

Figure 4: Relative expression of candidate metastasis-suppressor genes. Relative expression is normalized to GAPDH and MDA-MB-435.

re-amplified and were chosen for Northern blot screening.

These 18 cDNA fragments were gel purified, radiolabeled and used to probe screening Northern blots prepared from the panel of cells isolated from MDA MB 435 and its nonmetastatic neo11/MDA-MB-435 hybrids. Only seven (7) cDNA fragments produced the appropriate expression pattern and the results are shown in **Figure 3**. Comparison of expression was normalized to GAPDH and parental MDA-MB-435 following phosphor image densitometry. Relative expression is graphed in **Figure 4**.

Six of the seven candidate cDNA inserts were sequenced and homology assessed by comparing with the GenBank/ EMBL/ DDBJ/ PDB combined database. Three cDNAs were virtually 100% homologous to known human genes. The remainder were novel (**Table 3**). The three novel genes (temporarily designated 8A3, F5A3 and G1A2) were chosen for further characterization.

Table 3: Differentially expressed cDNAs isolated from neo11/MDA-MB-435 hybrids

cDNA	Size (bp)	% homology	Gene	Size on RNA blot
A1	387	99	GALNS [1]	8
1	370	99	APRT [2]	4.4
8A3 [3]	506	—	novel	1.2
F5A3 [3]	229	—	novel	1.5
G1A2 [3]	691	—	novel	1.3
G1B5	725	97	HHII [4]	3.2

[1] N-acetylgalactosamine 6-sulfatase; [2] Adenine phosphoribosyltransferase; [3] Candidates chosen for further analysis; [4] Hexokinase II

Since the novel cDNA fragments were isolated from cancer cells and were partial length, we wanted to determine whether they were expressed in normal tissues in order to minimize the probability that a mutant gene product was being pursued. Also, this information would be useful in determining which libraries were most suitable for obtaining full-length cDNAs. Highest level expression was observed for all three candidates in kidney (**Figure 5**). Since we had a placental library on hand and since expression was high for 8A3 in this tissue, we started isolations of full-length 8A3 first.

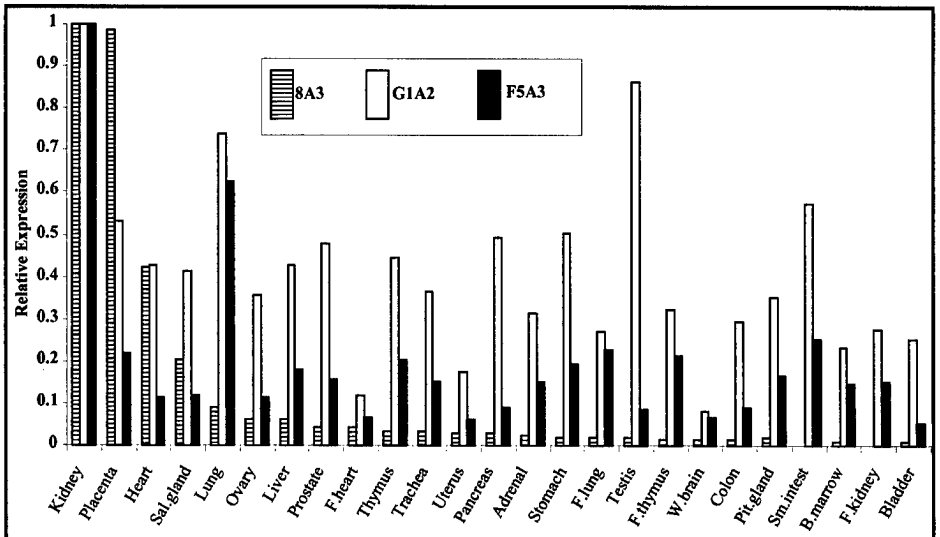

Figure 5: Expression of novel candidate genes in normal human tissue Northern blot.

Colonies were obtained and evaluated by Southern and Northern blots. Of six colonies, four produced the same size band in a Northern blot (1.2 kb). Although the loading is awful in this blot, the results show that the cDNAs are more highly expressed in neo11/MDA-MB-435 clones (**Figure 6**).

All of the cDNA clones were sequenced and aligned with the original 8A3 fragment as well as with each other. Four of the six clones matched 8A3 sequence completely and were larger, representing both the 5'- and 3'- flanking regions including the stop codon and poly (A) tail. Clones A1 and A2 were identical; however, the other two may be alternative splice variants of 8A3. As of July 13th, none of the four clones represent complete cDNA sequence (i.e., start codon with flanking Kozak sequence). The largest clone, to date, is 1.1 kb. Thus we appear to lack about 100 bp based upon Northern band size.

We recently began 5'-RACE to obtain the remaining cDNA. Gene specific primers were designed and using the placental cDNA library as a template, PCR was performed to amplify the 5'- end of the gene. Three PCR products were obtained, gel purified and transferred to a membrane and probed with 8A3 variants. A band of approximately 500 bp showed strong hybridization signal with the gene specific probe. Sequencing is currently underway.

A kidney cDNA library has been purchased and attempts have begun to isolate full-length F5A3 and G1A2.

Summary of findings and recommendations for follow-up experiments: Differential display represents the greatest "home run" approach; therefore, we will continue with this strategy. In year 1, Dr. Cheol Kyu Hwang did a lot of work on this project. However, none of his candidates panned out. Dr. Jabed Seraj re-started the differential display. Although progress has been slower than anticipated, he has identified three novel candidate cDNAs. By later 1998, we expect to have full-length cDNAs in hand for all three. To his credit, Dr. Seraj has overcome many of the previously unanticipated technical problems associated with this aim.

Section 6: **KiSS-1 is not overexpressed in neo11/MDA-MB-435 hybrids**

Since we previously showed that KiSS-1 is not expressed in parental, metastatic MDA-MB-435 cells and that KiSS-1 could suppress metastasis when transfected into these cells, we asked whether neo11/MDA-MB-435 cells express KiSS-1. Using Northern blotting, we found that the neo11/MDA-MB-435 cells do not express detectable levels of KiSS-1 mRNA. It is possible that the gene(s) product(s) encoded on chromosome 11 is acting downstream of KiSS-1; however, additional experiments will be

required to assess this possibility.

Section 7: **Overexpression of MEK1 transforms NIH3T3 cells and induces metastasis**

When presenting a talk at a Gordon Conference on our melanoma work, Dr. Alessandro Alessandrini noticed that KiSS-1 might contain a phosphorylation site of MEK1. Without presenting the year-long discussions that led to a formal collaboration, it became conceivable that MEK1 might be involved in controlling some aspects of metastasis. Briefly, the following observations led to this concept — (1) transfection of activated Ha-ras (upstream of MEK1 in many signaling schemes) into NIH3T3 cells renders them tumorigenic and metastatic; (2) transfection of MEK1 into NIH3T3 cells causes morphologic transformation; (3) variants of MEK1 with differential ERK activation potential are equally transforming *in vitro*; and (4) MEK transfectants express high levels of cathepsin L (a proteinase). The latter suggested that we ask whether the MEK1 transfectants are metastatic *in vivo*. Indeed, they were highly metastatic (abstracts appended). Moreover, we have apparently defined more completely the pathway(s) involved in activated ras-induced metastasis.

Section 8: **Protein kinase C δ potentiates growth in metastatic mammary cell lines**

After meeting at study section, Dr. Sue Jaken and I initiated a collaboration to study the role of protein kinases in breast cancer metastasis. This collaboration takes advantage of our respective experiences. Initially, we screened a series of rat mammary adenocarcinomas with varying metastatic potentials and found the most impressive change was increased expression/activity of the delta isoform. Two experiments were initiated to test the importance of this finding — transfection of PKCδ into poorly metastatic variants and determine whether metastatic potential increases and transfection of a dominant negative construct into highly metastatic variants and determine whether metastasis decreases. We are putting the finishing touches on the manuscripts but the predictions were correct within the 13762NF model system. Two manuscripts have been prepared (the first is appended). And we are following-up these observations using the human breast cancer models we have in hand.

PROGRESS AS RELATED TO STATEMENT OF WORK

Objective #1: Map the gene(s) responsible for suppressing metastasis of MDA-MB-435 to within 5 Mb by using MMCT with radiation-deletion variants of chromosome 11

Task 1-1 (Months 1-12): Identify polymorphic markers distinguishing MDA-MB-435 and donor chromosome 11

We have identified more than 30 polymorphic markers

Task 1-2 (Months 6-18): Prepare deletion variants of chromosome 11

Several chromosome 11 donors with deletions are in hand (Section 4)

Task 1-3 (Months 7-19): Prepare microcell hybrids with radiation deletion variants

This task has been initiated. Progress has been slower than expected. (Section 4). An alternative approach using PAC and BAC transfections is being considered as an alternative. The technician responsible for this objective has been replaced by a postdoctoral fellow.

Task 1-4 (Months 8-24): Confirm hybrids actually contain added chromosome 11 material by multiple criteria

This task has been initiated with the hybrids in -hand (Section 4).

Task 1-5 (Months 12-24): Test hybrids for metastasis in orthotopic metastasis model

One of the hybrids was tested. No suppression was observed in limited experiment (Section 4, Table 2)

Task 1-6 (Months 24-48): Repeat above in independent series

Task 1-7 (Months 24-26): Map deletions in hybrids (1st set), prepare map of overlapping

regions

Task 1-8 (Months 36-48): Map deletions in hybrids (2nd set), prepare map of overlapping regions

Objective #2: Stably introduce intact neo-tagged human chromosome 11 into MDA-MB-231 cells by MMCT

Task 2-1 (Months 1-6): Expand MDA-MB-231 cultures, verify pathogen-free (Mycoplasma free)

Completed first round of experiments, but none of the MDA-MB-231 variants were metastatic. Alternative strategies to obtain metastatic human breast carcinomas initiated. (Section 2)

Task 2-2 (Months 6-12): Prepare chromosome 11 hybrids

Not done, see below

Task 2-3 (Months 10-18):Confirm hybrids actually contain added chromosome 11 material by multiple criteria

Task 2-4 (Months 8-24):Test hybrids for metastasis in orthotopic metastasis model

Task 2-5 (Months 12-24): Prepare chromosome 6 and chromosome 15 hybrids, repeat metastasis study

Task 2-6 (Months 24-36):Confirm hybrids actually contain added chromosome 11 material by multiple criteria

Task 2-7 (Months 24-36): Test hybrids for metastasis in orthotopic metastasis model

Tasks 2-2 through 2-4 could not be done due to lack of appropriate models. We are attempting to obtain metastatic human breast carcinoma models in order to accomplish this important aim.

Objective #3: Identify metastasis-associated genes in neo11/MDA-MB-435 cells using differential display and/or subtraction hybridization

Task 3-1 (Months 6-12): Prepare cDNA library from neo11/435.B1 cells, Prepare "screening" RNA blots

Completed, additional libraries were prepared from mixtures of the neo11/MDA-MB-435 cells for use in subtractive hybridizations/differential display.

Task 3-2 (Months 6-9): Perform random primer amplification and repeat amplification for differential display

Completed. Initial experiments identified areas of concern using the human breast lines that were unanticipated. Therefore, pooling mRNAs to "normalize" the neo11/MDA-MB-435 were initiated.

Task 3-3 (Months 9-12): Perform "screening" Northern blots with probes from differential display

Completed four cycles. Six candidate genes identified and partial cDNA fragments sequenced. Follow-up with 3 novel cDNAs is proceeding.

Task 3-4 (Months 12-18): Sequence positive sequences, determine novelty, obtain full-length

Completed four cycles. Partial cDNAs sequenced and three novel genes were identified. Library screening has been done to identify a potential source for obtaining full-length cDNAs. Candidate cDNAs of appropriate length have been obtained for one of the three novel cDNAs. Sequencing is in progress. Library screening for the two other genes is in progress.

Task 3-5 (Months 18-24): Repeat Northern blots with longer probes for specificity

Completed four cycles and the pattern of expression warrants further study. Only the results which "make sense" are included in this report since many false positives were eliminated at prior stages.

Task 3-6 (Months 9-18): Prepare subtraction library

See Tasks 3-1 and 3-2. Subtraction library approach has been put on hold for now.

Task 3-7 (Months 18-30): Probe Northern blots with subtraction library

See Task 3-3

Task 3-8 (Months 36-48): Obtain full-length sequence for genes expressed in subtraction library

Progress has been slightly slower than anticipated. However, we are generally on target for the time line proposed. A new postdoctoral trainee has taken over this project and is making significantly better progress. We anticipate that transfection and evaluation of the candidate gene(s) will begin by 4Q98 or 1Q99.

Objective #4: Determine whether specific genes (such as KAI-1) is a metastasis-suppressor gene in MDA-MB-435 and MDA-MB-231 cells

Task 4-1 (Months 1-6): Prepare transfectants with KAI-1

Completed

Task 4-2 (Months 6-8): Select transfectants with increased KAI-1 expression

Completed

Task 4-3 (Months 9-18): Evaluate transfectants in orthotopic metastasis assay

Completed

Task 4-4 (Months 18-48): Prepare and evaluate transfectants prepared from genes isolated in Technical Objectives 1 and 3 above.

All four tasks have been completed and manuscripts published. This was made possible through collaborations with Drs. Barrett, Wei and Weissman, with whom we allied to study Kai-1 in breast cancer. We anticipate that at least one of the candidate genes identified in Objective 3 will be evaluated *in vivo* during 1Q99-3Q99.

CONCLUSIONS

Our preliminary data suggests that chromosome 11 encodes at least one human metastasis-suppressor gene. Our objective is to map and clone the gene(s) using parallel approaches. Approach 1 is to introduce pieces of chromosome 11, establish metastatic potential of the chromosome 11/breast cancer hybrids, map the gene(s) by regions of overlap. From July 1996-June 1998, we have established several baseline parameters necessary for completing this aim (i.e., identification of 30+ polymorphic markers that discriminate MDA-MB-435 and donor chromosome 11) and availability of characterized chromosome 11 donors with defined deletions). Unfortunately, the efficiency of MMCT has been horrible.

Approach 2 is to identify differentially expressed genes by differential display and/or representational differential analysis. Three rounds of screening using subtractive hybridization and differential display have been done. Three novel candidate genes have been identified and are being characterized.

Candidate genes identified using the approaches outlined above were then to be evaluated for their ability to suppress metastasis. Kai-1 was tested and found to inhibit metastasis, but the level of suppression did not instill confidence for follow-up. We showed that KiSS-1 could suppress metastasis of human breast carcinomas, but that KiSS-1 is apparently not a mediator of the chromosome 11 suppression.

Another objective was to determine whether our preliminary observations could be extended to other human breast cancer cells. Our plan was to introduce chromosome 11 by MMCT into MDA-MB-231. Unfortunately, the MDA-MB-231 cells, thus far, have not been metastatic. This was totally unexpected. Therefore, we are evaluating other variants of MDA-MB-231 as well as other human breast carcinoma cell lines.

The bottom line is that we have made progress toward completion of all four specific aims. Aim 4 was completed faster than expected, but progress on Aims 1-3 has been slower than anticipated. Nonetheless, we are continuing to make progress that approximates the time line proposed in the original proposal.

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APPENDICES

Transfection with constitutively active Mek1 confers tumorigenic and metastatic potential to NIH-3T3 cells

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Cell growth and differentiation are regulated by a variety of extracellular signals that are mediated by a family of serine/threonine kinases termed MAP (**M**itogen **A**ctivated **P**rotein) kinases or Erks (**E**xtracellular-signal **R**egulated **K**inases). Some components of the MAP kinase pathways, such as gip2, Ras, and Raf cause oncogenic transformation when constitutively active. Constitutively active Ras can confer metastatic potential upon some cells ([58]).

Activation of MAP kinases requires phosphorylation of both Thr and Tyr in a conserved "TEY" region of the catalytic domain. A family of dual-specificity kinases, called Meks (**M**AP kinase/**E**rk **K**inase), are responsible for this phosphorylation. Mek1 is activated by phosphorylation at Ser^{218} and Ser^{222} by Raf. Mutation of these two sites to acidic residues, particularly [Asp^{218}], [Asp^{218}, Asp^{222}] and

[Glu^{218}, Glu^{222}], results in constitutively active Mek1.

Using these mutant variants, we have previously shown that transfection of NIH/3T3 or Swiss 3T3 cells increases growth on soft agar ([59]). We also showed that growth of the [Asp^{218}] mutant did not correlate with Erk or Raf activity — [Asp^{218}] lines activate Erk1/2 but yield fewer colonies on soft agar. Even when dominant-negative Ras was introduced, Erk and Raf activities were not greatly affected. However, the same dominant negative construct introduced into v-src- or [Asp^{218}, Asp^{222}]-transformed cells caused severe reversion of src-expressing cells, but mild reversion of [Asp^{218}, Asp^{222}]-expressing cells. These data suggest that maintenance of *in vitro* transformation by Mek1 occurs through a Ras-independent pathway, and that the degree of transformation is independent of Raf1 and Erk1 activity.

NIH3T3 cells transfected with the [Asp^{218}] or [Asp^{218}, Asp^{222}] were tested for metastatic potential following intravenous injection into athymic mice. Parental cells formed no tumors grossly or histologically. However, all Mek1 mutant transformants formed macroscopic metastases. Thus, like Ras, Mek1 can confer both tumorigenic and metastatic potential upon NIH3T3 cells. These results refine the mechanism through which ras could confer tumorigenic and metastatic potential — i.e., the critical determinants of tumorigenic and metastatic potential are downstream of Mek1.

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Constitutively active MEK1 induces metastatic potential in NIH-3T3 cells

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Growth and differentiation are controlled by many extracellular signals, many of which activate the MAP kinase or Erk kinase families. Components of the MAP kinase pathways (e.g. gip2, Ras, Raf) cause oncogenic transformation in their constitutively active forms. However, MAP kinase activation occurs concomitant with PC12 differentiation induced by NGF.
MAP kinase activation requires the phosphorylation of both Thr and Tyr in the catalytic domain. A family of dual-specificity kinases called Meks (MAP kinase/Erk Kinase), are responsible for this phosphorylation and activation of MAP kinases. Mek1 is activated by phosphorylation on Ser 218 and 222 by Raf. Mutation of the serines, [Asp218] and [Asp218, Asp222], activates Mek1 constitutively. Stable expression of the constitutively active Mek1 mutants causes neuronal differentiation of PC12 cells and oncogenic transformation of fibroblast cell lines.
NIH 3T3 and Swiss 3T3 clonal cell lines expressing [Asp218] and [Asp218, Asp222] Mek1 mutants were made (Alessandrini et al., J Biol Chem. 1996. 271: 31612). Activated Mek1 causes transformation but is not correlated with Erk activity, i.e., [Asp218]-clonal lines yield fewer colonies on soft agar, yet exhibit constitutively active Erk1/2. The data suggest that maintenance of transformation by Mek1 mutants occurs through an ERK1/2-independent pathway, and that the degree of transformation is independent of Erk1 activity. Furthermore, these MEK1-infected NIH-3T3 clonal cell lines were metastatic to lungs following intravenous injection into athymic mice. Induction of metastatic also potential appears to be independent of Erk1/2 activity.

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Appendix
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Endocrine-related cancer
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Genetic and epigenetic regulation of human breast cancer progression and metastasis

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Introduction

Breast cancer is the most common malignancy and a major cause of cancer-related deaths among women in the United States and Western Europe (American Cancer Society 1998; Wingo, *et al.* 1998). Most women succumb to breast cancer if their tumors metastasize but cures are more likely if the cancers remain localized (Harris, *et al.* 1992a; Harris, *et al.* 1992b; Harris, *et al.* 1992c; Walker, *et al.* 1997). Thus, a greater understanding of the metastatic process in human breast cancer should translate into substantial improvements in therapeutic outcome for breast cancer patients. Toward that end, we will review and summarize the literature about, and begin to develop a working model for, the genetics of human breast cancer metastasis. There have been great strides in recent years with regard to our overall understanding of metastasis. Yet our apparently straightforward objective — to define cause-effect relationships for genes in breast cancer — was difficult because of four issues. First, many reports fail to distinguish between oncogenesis and progression or invasion and metastasis when reporting data. Second, there is a failure, by some, to recognize that breast cancer is not a single disease, but a collection of diseases. This is particularly apparent in the genetics literature. Third, it is difficult to evaluate the relative importance of correlative data, particularly as it relates to mechanistic control of steps in the metastatic cascade. Fourth, there is a tremendous noise-to-signal ratio for genetics of late-stage, metastatic breast cancers resulting from genotypic instability, phenotypic drift and tumor heterogeneity.

There are several assertions in the literature claiming a role for genes in controlling progression and/or metastasis of breast cancer. Out-of-hand dismissal for some of those claims was possible because the studies lacked necessary controls. For other genes, the data were more preliminary or correlative. And for an extremely small number of genes, functional data demonstrating regulation of breast cancer metastasis was available. The text of this review will focus on the latter; however, we decided that the utility of this article would be maximized if we summarized the known role(s) of individual breast cancer-associated genes, clearly discriminating the genes that regulate oncogenesis from those that control metastasis. The most effective method to accomplish this goal was to create tables that summarize the references providing evidence for a particular role(s) of genes in human breast cancer. Table 1 is designed to be used as a resource. Putative role(s) of individual genes in breast cancer are separated into two categories – oncogenesis and progression/metastasis – where key references are given to substantiate/refute a role. Although we attempted to be thorough and inclusive, the extensive historical literature combined with the rapidly evolving breast cancer genetics field limit the completeness of this review. We apologize to those whose work was not included because of space

considerations or whose papers were inadvertently omitted. However, we hope that this review fulfils our fourfold objective: (1) to highlight the genes for which roles in late-stage human breast cancer and/or metastasis have been functionally demonstrated; (2) to distinguish those genes from the more numerous oncogenic or tumor suppressors involved in breast cancer; (3) to evaluate the literature in order to identify needs for the field of breast cancer metastasis research to move to the next level; and (4) to propose a working model for the genetics of human breast cancer progression, focusing on the genes that have demonstrable metastasis-regulatory activity.

Breast cancer is a collection of diseases

Invasive breast cancers are a histologically and biochemically heterogeneous set of diseases. Lesions are typically categorized on the basis of histologic appearance, resembling either ductal or lobular components of the healthy breast. Most studies suggest that the majority of tumors arise in the terminal ductal unit of the breast, perhaps in a single type of "target" cell (Goehring & Morabia 1997; Russo & Russo 1997). By far, the most common type of breast cancer is infiltrating ductal carcinoma. This class of tumors represents nearly three quarters of all human breast cancers. Infiltrating lobular carcinomas account for 5-10% of breast carcinomas and are often characterized by multicentric tumors in the same or contralateral breast. Both ductal and lobular carcinomas have a predisposition for metastases to draining axillary lymph nodes, but each has differential predisposition for bone or visceral metastasis (Coleman, *et al.* 1998; Harris, *et al.* 1984). The molecular basis for these differences are mostly unknown. There are numerous other special types of invasive breast carcinomas. The most common are medullary, tubular and mucinous carcinomas. Medullary accounts for 5-7% of all breast carcinomas and are frequently well-circumscribed and exhibit lymphocytic infiltration (Fisher, *et al.* 1990). Mucinous (or colloid) carcinomas account for 1-3% of breast carcinomas and are characterized, as their name implies, by accumulation of mucin around the tumor cells. Overall prognosis for mucinous tumors is better than ductal or lobular carcinomas.

Based solely on their clinical behaviors, these are distinct types of breast carcinoma. It is likely that different genes are involved in controlling development and progression of each type. Yet most discussions of breast cancer genetics have not, for the most part, discriminated between each type of carcinoma. This is even more apparent when discussing the genetics of late-stage breast cancer. Since infiltrating ductal carcinomas are the most prevalent breast carcinoma type, most of the published results probably apply to ductal carcinomas, but this is not necessarily a good assumption (Afify, *et al.* 1996;

Larsson, *et al.* 1990; Nishizaki, *et al.* 1997; Toikkanen, *et al.* 1997). There is fortunately a recent trend towards studying cancer genetics using more refined pathologic criteria; however, more effort is required.

Further complications occur because of the use of cell lines which have been maintained in culture or passaged in animals for several years. The cells have probably undergone genotypic and phenotypic drift as well as selection pressures so that resemblance of the cell lines to the original tumor may be minimal. Sadly, though most breast carcinoma cell lines were derived from metastatic lesions, most no longer retain this ability in experimental systems (i.e., metastasis from mammary fat pads in immunocompromised (athymic or SCID) mice). This limitation severely hinders the ability of investigators to directly assess the metastasis-regulatory effects of individual genes. Given these caveats, any generalizations should be viewed with healthy skepticism. Nonetheless, certain patterns emerge and allow us to make a reasonable first approximation for a model of the molecular underpinnings of breast cancer progression and metastasis.

Oncogenesis and tumor progression are linked, but distinct, phenotypes

One area of confusion relates to terminology. Sloppy use of, and dual meanings of, some terms (depending upon one's specialization) are prevalent in the literature. Of particular relevance to this review are the distinctions between tumorigenesis *vs.* tumor progression and malignant *vs.* metastatic. Tumorigenesis and oncogenesis refer to the ability of cells to proliferate continuously in the absence of persistent stimulation by the triggering agent(s). Tumor progression is the evolution of already tumorigenic cells (populations) towards an increasingly autonomous state (i.e., decreased dependence upon host-derived growth factors and/or increased resistance to negative regulatory molecules). The distinction between oncogenesis and progression is crucial when asking whether a gene is important in controlling steps associated with malignancy, as compared to whether that gene is involved in tumor formation.

The distinctions between malignant and metastatic are more subtle. Attributes of malignant cells include (but are not limited to) less differentiated morphology, less differentiated cytology, level of vascularity, level of necrosis, mitotic index, aneuploidy, nuclear:cytoplasmic ratio. The incontrovertible hallmarks of malignancy are invasion of cells though a basement membrane and/or metastasis. All other characteristics used to label a tumor (and the cells within it) as malignant have exceptions (Pfeifer & Wick 1995). For example, morphologically indolent cells may be behaviorally malignant and *vice versa.*

Clearly, parameters associated with pathologic examination are invaluable when estimating the probability for local, regional or distant recurrence in a clinical setting. Nonetheless, subjectivity leads to ambiguity when trying to assign responsibility for a phenotype (i.e., metastasis).

Metastasis is defined as the formation of secondary tumor foci discontinuous from the primary tumor. The metastases can be nearby or at distant sites. Metastases can form following dissemination of cells via lymphatic, hematogenous, coelomic cavities or epithelial cavities. Since they are, by far, the most common routes for metastatic spread of human breast cancer, lymphatic and hematogenous metastasis will be the focus here. In order to metastasize, cells must complete every step of a complex cascade. Malignant cells invade adjacent tissues and penetrate into the lymphatic and/or circulatory systems. Then tumor cells detach from the primary tumor and disseminate. During transport, cells travel individually or as emboli composed of tumor cells (homotypic) or tumor cells and host cells (heterotypic). At a secondary site, cells or emboli either arrest because of physical limitations (e.g., too large to traverse a capillary lumen) or by binding to specific molecules in particular organs or tissues. Once there, tumor cells then proliferate either in the vasculature or extravasate into surrounding tissue (Chambers, *et al.* 1995; Koop, *et al.* 1996). To form macroscopic metastases, cells must then recruit a vascular supply (Ellis & Fidler 1995; Folkman 1995; Kohn & Liotta 1995; Weinstat-Saslow & Steeg 1994a) and respond appropriately to the tissue's environmental milieu (Nicolson 1994; Radinsky 1995). Fewer than 0.1% of cells that enter the vasculature survive to form clinically detectable, macroscopic metastases (Fidler 1970; Tarin, *et al.* 1984). At which step(s) of the metastatic cascade circulating tumor cells commonly succumb is debatable (Chambers, *et al.* 1995; Koop, *et al.* 1995; Koop, *et al.* 1996).

In the context of a multistep, multigenic cascade, it is critical to recognize that the terms *invasiveness* and *adhesion* are not equivalent to metastatic propensity. Both invasion and adhesion are necessary, but not sufficient for metastasis. Cells that are efficient at either or both — but which lack the ability to complete any other step of the metastatic cascade — are nonmetastatic (Fidler & Radinsky 1990). Therefore, correlations of genetic expression to a particular step in the metastatic cascade may lead to erroneous conclusions.

Taken together, these points emphasize the importance for distinguishing *tumor*-suppressor and *metastasis*-suppressor genes. The former dominantly inhibit tumor formation when wild-type expression is restored in a neoplastic cell. By definition, then, metastasis would also be suppressed (since the cells are nontumorigenic). Metastasis-suppressor genes, on the other hand, block only the ability to form metastases. Restoring expression of a metastasis-suppressor would yield cells which are still tumorigenic,

but are no longer metastatic.

At diagnosis, breast carcinomas are typically mixtures of genotypically and phenotypically distinct cells despite having arisen from a single cells (Fujii, *et al.* 1996a; Fujii, *et al.* 1996b; Rebbeck, *et al.* 1996; Shows, *et al.* 1997; Welch & Tomasovic 1985). One of the earliest detectable changes in transformed (anchorage independent, not contact inhibited, immortal but not necessarily able to form a tumor in an appropriate host) cells is a several-fold increase of genomic instability compared to normal cells (Ling, *et al.* 1985; Tlsty 1997; Tlsty, *et al.* 1993; Cheng & Loeb 1993). Karyotypic and genomic instability is present in transformed cells even before they acquire tumorigenic potential (Jonczyk, *et al.* 1993; Otto, *et al.* 1989; Tlsty 1990; Tlsty, *et al.* 1993). Thus, genomic instability appears to be the driving force by which cells acquire the cumulative genetic defects necessary to be fully tumorigenic. Likewise, the development of heterogeneity, coupled with selective pressures results in continued evolution of the tumor population, usually toward increasing autonomy from the host (Foulds 1954; Heppner 1984; Heppner & Miller 1997; Welch & Tomasovic 1985; Welch & Tomasovic 1985). Eventually, some subpopulations of cells within the mass are amply self-sufficient that they have the ability to metastasize. This does not imply that metastatic cells do not respond to host-derived growth signals. Rather, it means that they do not necessarily require them. In conclusion, oncogenesis is a prerequisite for metastasis formation. In other words, metastatic cells represent a subset of tumorigenic cells.

One measure of genetic instability is microsatellite instability. Several reports have suggested that microsatellite instability is a useful prognostic indicator for breast cancer (Patel, *et al.* 1994; Paulson, *et al.* 1996; Yee, *et al.* 1994); however, a role in development of metastasis has not been established. Recently, another means for developing genetic instability in non-HNPCC colorectal cancers was described (Cahill, *et al.* 1998). Defective segregation machinery results in unequal partitioning of chromosomes in daughter cells, leading to aneuploidy. While it is common for breast carcinomas to be aneuploid, it has not yet been determined whether a similar mechanisms is taking place in breast. Regardless of mechanism, genetic instability has practical consequences with regard to our ability to isolate and characterize metastasis-associated genes — key genetic changes is sometimes clouded by background "noise" due to heterogeneity. Techniques such as tissue microdissection are now being utilized to minimize this problem (Zhuang, *et al.* 1995).

Therefore, the ability to establish a role for a given gene in breast cancer metastasis is complicated by a variety of factors. The following discussion will focus on those genes for which genetic

manipulation has been utilized to establish a role in controlling metastasis. Largely, the results are based upon experimental systems. Combined with clinical correlations, there is substantial evidence for controlling the metastatic potential of breast carcinoma.

The use of knockout and transgenic mice to study various aspects of breast cancer biology has been increasing in recent years (reviewed in (Amundadottir, *et al.* 1996; Bennett & Wiseman 1997; Clarke 1996; Li, *et al.* 1998; Thomas & Balkwill 1994)). The use of such models has focused on tumor development rather than the latter stages of tumor progression and metastasis. And while improvements are occurring at a rapid rate, the models are still limited by relatively poor mimicry of the pathogenesis of human breast cancer.

Metastasis-controlling genes in breast carcinoma

Since a working model for tumorigenesis involves mutations of key genes that control cell growth and/or death, it appears plausible that metastasis will also be controlled by a select set of genes controlling key steps in the cascade. Based upon this presumption, we will focus on genes that appear likely to be important in either the suppression (or promotion) of breast cancer metastasis. In this regard, the genetic basis of metastasis would parallel the genetics of tumor formation. Evidence shows that metastasis involves numerous genes (Chambers & Matrisian 1997; Fidler & Radinsky 1990; Price, *et al.* 1997; Welch & Goldberg 1997) that fall into two categories — (1) genes that drive metastasis formation, and (2) genes that inhibit metastasis (De La Rosa, *et al.* 1995; Dear & Kefford 1990; Dong, *et al.* 1995; Lee, *et al.* 1996; Lee & Welch 1997c; Phillips, *et al.* 1996; Welch, *et al.* 1994). The number of identified metastasis-associated genes are growing rapidly. However, their mechanisms of action, their regulation in normal and/or cancer cells, and the universality of function in cancers of different origin remains largely unknown.

The best characterized dominantly acting metastasis gene (i.e., met-oncogene, drives conversion from benign to malignant is the activated ras oncogene (Chambers, *et al.* 1990; Collard, *et al.* 1987; Phillips, *et al.* 1990). Transfection and constitutive expression of nonsenescent rodent fibroblasts with activated Ha-ras leads to development of tumorigenic and metastatic properties (Egan, *et al.* 1987; Muschel, *et al.* 1985). However, complete induction of metastasis does not occur in all cell lines or cell types (Chambers, *et al.* 1990; Jessell & Melton 1992; Tuck, *et al.* 1990), nor is retention of ras oncogene expression necessary to maintain the metastatic phenotype (Schlatter & Waghorne 1992). In human breast cancer, overexpression of normal or mutant ras in human breast cancer has been associated with

increased malignant properties (e.g., reduced responsiveness to estrogens, increased invasiveness, morphological abnormalities (Fromowitz, *et al.* 1987; Lundy, *et al.* 1986; Theillet, *et al.* 1986)), but association with metastatic potential has not been unequivocally demonstrated. Mutations of ras, per se, are relatively uncommon in human breast cancer; so, the importance of ras in controlling breast cancer metastasis is not completely understood.

The prototypical metastasis-suppressor gene, Nm23, was first identified in the murine K1735 melanoma using subtractive hybridization and its expression is inversely correlated with lung colonization (Bevilacqua, *et al.* 1989; Steeg, *et al.* 1988); but, there are exceptions (Radinsky, *et al.* 1992). The human homolog, *Nm*23-H1 [also known as NME1], exhibits decreased expression in late-stage, metastatic human breast, endometrial, ovarian, melanoma and colon cancers (reviewed in (Freije, *et al.* 1996)). However, long-term prognostic value has been questioned in some studies (Kapranos, *et al.* 1996; Russell, *et al.* 1997). Nonetheless, NME1 is a *bona fide* metastasis-suppressor gene in human breast carcinoma since transfection of metastatic MDA-MB-435 cells resulted in a significant suppression of metastasis from the mammary gland in experimental mouse models (Leone, *et al.* 1993). The mechanism of action for NME1 remains unknown (De La Rosa, *et al.* 1995), but motility of the transfectants was significantly suppressed (Kantor, *et al.* 1993). NME1 is homologous to *Drosophila awd* and encodes a 17 kDa protein. NME1's nucleoside diphosphate kinase homology (Biggs, *et al.* 1990) and function (Steeg, *et al.* 1991) have recently been dissociated from its metastasis-suppressor function (De La Rosa, *et al.* 1995; MacDonald, *et al.* 1993; Royds, *et al.* 1994). Some recent reports suggest that NME1 may be involved in controlling cell cycle progression (Cipollini, *et al.* 1997) and histidine-dependent protein phosphorylation reactions (Freije, *et al.* 1997).

The story for Nm23 becomes more complicated because three additional family members (Nm23-H2/NME2, Nm23-DR, Nm23-H4) have recently been identified and cloned. NME2 has transcriptional regulatory properties for *c-myc* (Berberich & Postel 1995; Ji, *et al.* 1995; Postel, *et al.* 1993; Seifert, *et al.* 1995). Some studies have shown that NME2 can suppress metastasis (Engel, *et al.* 1993; Mandai, *et al.* 1994; Marone, *et al.* 1996); whereas, others have not (Arai, *et al.* 1993; Baba, *et al.* 1995; Tokunaga, *et al.* 1993; Yamaguchi, *et al.* 1994). Nm23-DR is differentially expressed during myeloid differentiation (Venturelli, *et al.* 1995) but association with metastatic potential has not yet been tested in either clinical samples or experimental systems. Nm23-H4 differs structurally from the other homologs in that it appears to have additional N-terminal basic amino acid residues (Milon, *et al.* 1997). However, its mechanism of action and relevance to breast cancer biology have not yet been reported.

A recent study even suggests that expression levels of Nm23-H1 in human breast cancer cell lines (HT115 and MDA-MB-231) can be influenced by diet. Increased consumption of linoleic and arachidonic acids reduced expression whereas linolenic acid increased expression (Jiang, *et al.* 1998). These conditions lowered invasiveness as measured by *in vitro* invasion assays. While a significant amount of work needs to be done to determine whether dietary regulation of metastasis is mediated through modulation of Nm23, dietary fat intake has been shown to control breast and mammary tumor metastasis (Hubbard & Erickson 1987; Rose, *et al.* 1994; Rose, *et al.* 1995).

KAI1 (also known as CD82 or C33, members of the TM4SF superfamily of adhesion molecules) was recently discovered as a prostate cancer metastasis-suppressor gene on the p-arm of chromosome 11 (Dong, *et al.* 1995). Other members of the TM4SF family, namely MRP-1/CD9 and CD63/ME491, have been associated with metastatic potential of non small-cell human lung carcinomas (Ikeyama, *et al.* 1993) and early stage melanomas, (Hotta, *et al.* 1988), respectively. Thus, a role for KAI1 in breast cancer metastasis was possible. To test this hypothesis, we measured KAI1 mRNA expression in a panel of human cell lines representing a continuum from nearly normal breast cells (MCF10A) to highly metastatic cells (MDA-MB-435). KAI1 mRNA expression decreased with increasing invasive and metastatic potentials (Yang, *et al.* 1997).

Lower KAI1 expression in metastatic breast cancers correlated well with previous findings that chromosome 11 deletions are common in late-stage breast carcinoma (Devilee & Cornelisse 1990; Devilee & Cornelisse 1994a; Mars & Saunders 1990; Negrini, *et al.* 1995; Trent, *et al.* 1995). To directly test whether changes on chromosome 11 were responsible for suppressing metastatic potential, we introduced a normal chromosome 11 into metastatic MDA-MB-435 breast carcinoma by microcell-mediated chromosomal transfer. Chromosome 11 significantly reduced the metastatic properties without affecting tumorigenicity (Phillips, *et al.* 1996). Since KAI-1 expression was higher in the chromosome 11 hybrids, we hypothesized that KAI1 is the gene responsible for suppressing metastasis. Expression of another TM4SF family member, TAPA-1 which is also encoded on chromosome 11, did not correlate with metastatic potential. Transfection and stable constitutive expression of KAI1 in MDA-MB-435 cells suppressed metastasis from tumors following injection into the orthotopic site – mammary fat pad (Phillips, *et al.* 1998). However, the cell lines did not maintain transgene expression levels following *in vivo* growth. This complicated interpretation. Preliminary studies using a panel of human breast specimens of varying grade indicate that KAI1 protein staining was related inversely to grade of disease (Wang & Wei, unpublished observations). Nonetheless, KAI1 appears to meet the criteria described

above for metastasis-suppressor gene in human breast cancer.

Chromosome 1q deletions occur with variable frequency in late-stage human breast carcinomas. Since the recently discovered melanoma metastasis-suppressor gene, KiSS-1, maps to chromosome 1q32 (Lee, *et al.* 1996), we tested whether KiSS-1 could suppress metastasis of the human breast ductal carcinoma cell line MDA-MB-435. Parental MDA-MB-435 cells did not express KiSS-1; but nonmetastatic MDA-MB-231 breast carcinoma cells did. Transfection of a full-length, constitutive mammalian expression construct suppressed metastasis of MDA-MB-435 from the mammary fat pad of athymic mice; whereas, vector-only transfectants were unaffected (Lee & Welch 1997c).

The mechanism of action for KiSS-1 has not yet been determined although its ability to suppress metastasis has been demonstrated in six independently-derived human cancer cell lines of melanoma and breast origin (Lee, *et al.* 1997a; Lee & Welch 1997b; Lee & Welch 1997c). Based upon the cDNA sequence, the predicted KiSS-1 protein would be a hydrophilic, 164 amino acid protein with molecular mass of 15.4 kDa. The sequence is novel, having no strong homology to any known human cDNA sequences. Four regions within the predicted KiSS-1 protein match consensus as phosphorylation sites for protein kinase C, protein kinase A and a tyrosine kinase (Lee, *et al.* 1997a). These sequences suggest that KiSS-1 is a phosphoprotein and our working hypothesis is that it functions within a signal transduction pathway. Thus far, KiSS-1 expression has never been detected in any cells that have metastatic potential. However, all studies have measured mRNA expression since antibodies are not yet available. This deficiency limits our ability to measure clinical correlations, although this is certainly a high priority goal.

Other metastasis-promoting or invasion-promoting genes have identified in a variety of human and rodent tumor models. The genes include — TIAM-1 (Habets, *et al.* 1994), mts1 (Grigorian, *et al.* 1994), mta1 (Toh, *et al.* 1994),TI-241 (Ishiguro, *et al.* 1996), fibroblast growth factor-4 (Dickson & Lippman 1992; McLeskey, *et al.* 1996), and cathepsin D (Rochefort, *et al.* 1990a; Rochefort, *et al.* 1990b). Transfection of these genes into experimental cell systems (usually fibroblasts) is reported to increase invasiveness and metastasis. Again, definitive roles of these genes in mammary or breast cancers are not well-defined.

Protein kinase C (PKC) activities are important for several physiological processes relevant to mammary tumor promotion and progression (e.g., proliferation, motility, anchorage-independent growth, responses to growth factors, etc.). In collaboration with Drs. Susan Jaken, Sue Kiley and Daniel Medina,

we recently compared PKC isoenzyme levels in mouse and rat mammary tumor cell lines (Jaken, *et al.* 1997; Kiley, *et al.* 1996; Kiley, *et al.* 1998). Of particular relevance to this review, 13762NF mammary adenocarcinoma cell clones that have low, moderate and high metastatic potentials were evaluated for expression of PKCs α, δ, ϵ and ζ. All isoforms were expressed in each of the cell lines; however, PKCδ was significantly greater in highly metastatic compared to poorly metastatic cells. To determine whether this correlation was physiologically relevant, transfections were done to increase (full-length PKCδ cDNA in constitutive and inducible expression constructs) or decrease (dominant negative PKCδ regulatory domain (RDδ) in inducible expression constructs) PKCδ expression. Increased expression of PKCδ enhanced clonogenicity in soft agar and metastatic potential, but did not affect anchorage-dependent growth. Expression of the RDδ inhibited metastasis when cells were injected into syngeneic rats. Moreover, induction of the RDδ with doxycycline (which induces the tetracycline-inducible promoter) caused a significant reduction in metastatic potential. Taken together, our results strongly imply that PKCδ is an important regulator of mammary tumor metastasis. Experiments are underway to determine relevance of RDδ in controlling human breast cancer metastasis.

Chromosomal changes in breast cancer may predict the location of metastasis-controlling genes

As alluded above, consistent, non-random rearrangements, deletions and/or amplifications have been instrumental in identifying oncogenes and tumor-suppressor genes involved in the development of human cancer. Over 56 distinct regions of loss of heterozygosity (LOH) have been identified in breast cancer {Kerangueven, Noguchi, et al. 1997 ID: 9924}. The frequency of involvement of each ranges from <20% to >50% depending upon the study, tumor type and markers used. Unfortunately, as tumors progress, they accumulate changes, leading to complex karyotypes. Structural or numerical aberrations for virtually every chromosome have been described in human breast cancer (See Table 2 for an example). Experience has told us that some of the chromosomal changes occur at a frequency higher than could be explained on a random mutational basis. These findings increase the probability that genes associated with tumor progression will be encoded at those sites. LOH has been found in the following chromosomal regions correlating with parameters associated with breast cancer progression/metastasis — 1p and nodal status (Borg, *et al.* 1992b); 1q21-q24 and stage (Devilee, *et al.* 1991); 3p21-p25 and LOH on 11p, 17p, 17q and aneuploidy (Devilee, *et al.* 1994b); 7q23 and metastasis-free overall survival (Bieche, *et al.* 1992); 8p21.3-p23 in low grade DCIS (Anbazhagan, *et al.* 1998); 9q and LOH on 1q, 17p, 18q (Devilee, *et al.* 1994b); 11p15 and ER^- tumors, grade III tumors, and distant metastasis (Ali, *et al.*

1987); 11p15 and lymph node status (Takita, *et al.* 1992); 13q12-q14 and ER content (Devilee, *et al.* 1994b); 13q12-q14 and ductal carcinoma tumor size (Andersen, *et al.* 1992); 13q12-q14 and aneuploidy and S-phase fraction >12% (Borg, *et al.* 1992b); 16q24 and ER content (Devilee, *et al.* 1994b); 17q12-q24 and c-erb-B2 amplification (Sato, *et al.* 1991); 17q12-q24 and age of onset (Devilee, *et al.* 1994b); and 17q12-q24 and c-erb-B2 amplification / post-menopausal status (Andersen, *et al.* 1992). To emphasize the point made above – i.e., that different types of breast cancers exhibit different chromosomal changes – Nishizaki and colleagues used the comparative genomic hybridization technique to compare lobular and ductal carcinomas. Lobular carcinomas had increased copies of DNA from chromosome 1q in 79% of patient samples and losses of chromosome 16q in 63%. The lobular carcinomas showed higher frequency of 16q loss than ductal carcinomas and lower frequency of 8q and 20q gains (Nishizaki, *et al.* 1997).

In metastases *vs.* primary tumors, karyotypic abnormalities of chromosomes 1, 6, 7, and 11are particularly prevalent. Among the more common cytogenetic changes in metastases from breast is amplification in the region surrounding band q13 on chromosome 11. The amplicon includes the following genes: int-2 gene (which is syntenic to a site of frequent mouse mammary tumor virus (MMTV) insertional mutagenesis in mice (Lee, *et al.* 1995) but the protein is not usually expressed in human breast tumors); hst (which is a member of FGF family but this is not expressed at mRNA level (Nguyen, *et al.* 1988; Theilet, *et al.* 1989)); bcl-1 (which was discovered by involvement in chromosomal translocations in some lymphomas (Theillet, *et al.* 1990; Tsujimoto, *et al.* 1984)); and PRAD-1 (which was initially discovered in parathyroid adenomas(Motokura & Arnold 1993; Motokura, *et al.* 1991), but subsequently found to be cyclin D1 (Motokura & Arnold 1993; Motokura, *et al.* 1991)). Amplification in this region is associated with poor prognosis (Lidereau, *et al.* 1988; Tsuda, *et al.* 1989), presence of lymph node metastases (Adnane, *et al.* 1991; Theilet, *et al.* 1989; Zhou, *et al.* 1988), ER and PR status (Borg, *et al.* 1991; Fantl, *et al.* 1990; Theilet, *et al.* 1989). While these correlations are compelling, definitive association of 11q13 amplification with metastatic potential has not been demonstrated.

As mentioned above, microcell-mediated chromosomal transfer of chromosome 11 reveals that there exists a metastasis suppressor activity on chromosome 11. However, these types of experiments are complicated because results vary according to the experimental models used. Microcell transfer into MCF7 breast cancer cells revealed that BrCa-1- and p53-independent growth inhibitors (i.e., inhibitors of tumorigenicity) are encoded on chromosome 17 (Casey, *et al.* 1993; Plummer, *et al.* 1997; Theile, *et al.*

1995). Additional growth inhibitors have been described on chromosomes 6 and 11 (Negrini, *et al.* 1994; Shows, *et al.* 1997; Theile, *et al.* 1996). Interestingly, transfer of chromosome 11 suppresses growth in culture and tumor formation in the MDA-MB-231 and MCF7 models, but neither phenotype was significantly, nor consistently affected in MDA-MB-435. These data clearly show that extrapolation based upon data from a single model is ill-advised. However, this problem is not easily solved because of the problem mentioned above – lack of relevant metastatic models of human breast cancer.

Inadequate models exist to study breast cancer metastasis

Despite the fact that the majority of human breast cancer cell lines have been derived from metastatic lesions, only MDA-MB-435 reproducibly forms macrometastases when evaluated in athymic or SCID mice (Price 1996; Price, *et al.* 1990). This is a serious limitation for investigators wishing to study metastasis of human breast cancer. Several investigators have found that MDA-MB-231 will form lung metastases following injection into the mammary fat pad (Price, *et al.* 1990; Rose, *et al.* 1994) or bone metastases following intracardiac injection (Guise 1997; Mbalaviele, *et al.* 1996). Interestingly, none of the models currently available metastasize to bone following tumor growth in the mammary fat pad, despite this being the most common site for metastasis in clinical breast cancer (Coleman 1997). Three points deserve emphasis. First, lung colonization efficiency is generally lower in MDA-MB-231 than from MDA-MB-435. If metastasis suppression is the desired biological endpoint, it is important that baseline levels be as high as possible. Second, as with MCF7 cells, there are several different sublines of MDA-MB-435 and MDA-MB-231 that have been artificially selected over the years in may different labs. Some of these cells are no longer tumorigenic in immunocompromised mice. Therefore, it is incumbent upon each investigator to verify metastatic potential in his/her laboratory. Third, the distribution of metastatic lesions in immunocompromised mice does not completely mimic the clinical situation. While not inappropriate, the models are somewhat lacking in this regard.

Breast cancer metastasis is not solely due to genetic changes

A heritable component of the metastatic phenotype has been demonstrated numerous times by experimental isolation of metastatic and nonmetastatic clones as well as selection of increasingly metastatic variants from heterogeneous tumor populations. For cells to successfully metastasize, they must also interact with a variety of host cells and their secreted molecules and respond appropriately. Thus, any discussion of factors controlling metastasis must include an evaluation of exogenous regulators

of the process (or its component steps). Normal breast tissue growth, differentiation and regression after lactation are all exquisitely controlled by hormones. Indeed initiation, promotion and progression of breast carcinomas are strongly regulated by endocrine mechanisms (Dickson, *et al.* 1993; Kaufmann 1997).

Hormone contribute to breast cancer development and metastasis

Hormones have long been implicated for playing roles in the initiation, development, and progression of breast cancer. Numerous epidemiological studies spanning almost two decades have established that, excluding a genetic predisposition, the reproductive history of a women is an important risk factor associated with the development of breast cancer. Early menarche and late menopause have been shown to be associated with an increased risk of breast cancer. Epidemiological studies also show that early pregnancy provides a protective effect against breast cancer, but that the protection declines as the age of first pregnancy increases. Taken together, these studies suggest that the length of time between menarche and menopause or menarche and first pregnancy are contributing factors toward the risk or likelihood of breast cancer oncogenesis (Henderson, *et al.* 1991; Key & Pike 1988; Staszewski 1971).

The two principal hormones involved in both the onset of menarche and in menopause are the female sex steroids, estrogen (specifically 17β-estradiol) and progesterone. It is well-established that estrogen promotes breast cancer by stimulating cell division. Although the main source of estrogen is ovary in premenopausal women, estrogen can also be synthesized directly in adipose tissue and breast cancer cells via the enzyme aromatase {Yue, Wang, et al. 1998 ID: 11029}. Aromatization is typically thought to be the predominant source of estrogens in post-menopausal women (Brodie & Santen 1994; Harvey 1997; Kaufmann 1997). More controversial is the role that estrogens or estrogen metabolites can have in causing or initiating breast cancer. Recent findings suggest that metabolites of 17β-estradiol may be among the culprits leading to DNA damage and subsequently for initiation of breast cancer (Cavalieri, *et al.* 1997; Fishman, *et al.* 1995; Lavigne, *et al.* 1997; Zhu & Conney 1998). However, this interpretation is debatable and additional research will be required to establish this definitively. Nonetheless, there is little doubt that estrogens play a key role in promoting initiated human breast cancer to grow and to progress.

A role for progesterone in breast cancer development is less clear than for estrogen. At one time, it was generally accepted that progesterone was a natural antagonist of estrogen action — suggesting that it would inhibit or block growth promoting effects of estradiol on breast cells (normal and tumor). This

paradigm was based upon findings in the uterus in which progestins reduced or eliminated the risk of estrogen-induced endometrial cancer. Recently, the effect of progesterone (analogs) on normal breast epithelial cells has been re-examined. The mitotic index of normal breast epithelial cells parallels changes in hormone levels during the menstrual cycle. In cycling women, serum estrogen levels are highest during the follicular phase with a secondary resurgence in the secretory phase. The mitotic index of endometrial cells parallels serum estrogen levels. In contrast, breast epithelial mitoses are greatest during the secretory phase when serum progesterone levels are maximal (Going, *et al.* 1988; Masters, *et al.* 1977; Meyer 1977). The latter raises the possibility that progesterone may have growth promoting effects on breast epithelial cells. This supposition is further supported by the following lines of evidence: (1) progestins are mitogenic for established breast cancer cell lines *in vitro* (Hissom, *et al.* 1989; Hissom & Moore 1987; Manni, *et al.* 1991); (2) progestins promote growth of established mammary tumors (Huggins 1965; Huggins & Yang 1962; Robinson & Jordan 1987); (3) progestins stimulate expression of mitogenic growth factors and/or their receptors (Dickson & Lippman 1988; Lanari, *et al.* 1989; Murphy & Dotzlaw 1989; Murphy, *et al.* 1988; Papa, *et al.* 1991); and (4) anti-progestins induce apoptosis in experimental mammary tumor models (Michna, *et al.* 1989; Schneider, *et al.* 1989). Thus, progesterone exposure may be a contributing factor toward the development of breast cancer.

Estrogen and progesterone exert their cellular effects through interactions with nuclear receptor proteins called the estrogen receptor (ER) and progesterone receptor (PR), respectively. The recognition that these receptors are the primary mediators of estrogen and progesterone action and that their presence within a tumor specimen can help predict the responsiveness of human breast cancer to hormonal therapy is particularly useful. Today, the measurement of ER levels is standard practice and is a useful prognostic marker in determining which patients are most likely to respond to estrogen antagonist therapies such as the antiestrogen, Tamoxifen (also known as Nolvadex). Since PR is an estrogen-induced product, simultaneous detection of PR in the presence of ER from a single tumor is indicative of a functional estrogen receptor pathway and further improves the ability to predict response to antiestrogen therapy. Alternatively, the absence of ER and PR is associated with early recurrence and poor survival of the breast cancer patient.

The ER mentioned above refers to the alpha ER (ER-α). Recently, a second ER form has been cloned (ER-β) (Kuiper, *et al.* 1996). ER-α and ER-β both bind 17β-estradiol in traditional binding assays. However, current data suggest that the amount of ER-β relative to ER-α in breast cancer cells is minor (Kuiper, *et al.* 1996; Petersen, *et al.* 1998). In the normal mammary glands of mice, ER-β is

undetectable (Couse, *et al.* 1997). Whether ER-β will play an important role in breast cancer biology or etiology remains to be determined; although there have been reports of ER-β mutants in breast cancer cells (Dotzlaw, *et al.* 1997; Vladusic, *et al.* 1998).

Since almost all breast cancers progress from a hormone-responsive state to a hormone-resistant or hormone non-responsive state, the possibility was raised that mutations in the ER-α (the predominant form of ER is breast cancers) could be a factor leading to antiestrogen resistance in breast cancer. Several investigators pursued this line of thought and have shown that mutant ER exist in some breast cancer cell lines and tumor specimens (Fuqua, *et al.* 1992; Fuqua, *et al.* 1991a; Graham, *et al.* 1990; Scott, *et al.* 1991; Wang & Miksicek 1991). Moreover, mutations of ER can lead to variant estrogen receptor activity which, in turn, may explain estrogen resistance (Fuqua, *et al.* 1991a) (Fuqua, *et al.* 1992). Furthermore, these and other studies that have focused on ligand-receptor interactions, it is apparent that variations in ER structure and ligand specific (estrogen versus antiestrogen) interactions with ER may lead to altered and unexpected biological responses (Katzenellenbogen 1996; Levenson, *et al.* 1997; McInerney & Katzenellenbogen 1996; Montano, *et al.* 1996). This is further complicated by promoter and cell-specific factors (Katzenellenbogen 1996; Yang, *et al.* 1996). Although the existence of mutant ER is very appealing, their actual contribution to disease progression, particularly antiestrogen resistance, appears to be small. Furthermore, most of the variant ER data to date has been found at the mRNA level. It is still not known whether they are translated into proteins (Dowsett, *et al.* 1997; Murphy, *et al.* 1997a; Murphy, *et al.* 1997b; Tonetti & Jordan 1997).

Although less research has been dedicated toward the identification of variant PR, there are several papers reporting the existence of variant PR mRNA and protein (Leygue, *et al.* 1996a; Wei, *et al.* 1990; Wei & Miner 1994; Yeates, *et al.* 1998). One variant PR protein form is N-terminally truncated compared to the previously reported A- and B- PR isoforms. This third form, the so-called C-receptor, has unique transcriptional enhancing properties when in the presence of the two larger PR isoforms and ligand (Wei, *et al.* 1996). From this work and the abundance of other studies, it is becoming apparent steroid-regulated growth and gene expression involves multiple regulatory factors, of which the steroid receptor is but one component, and that the eventual biological outcome is dependent upon the interaction of steroid receptors with non-receptor proteins (i.e., adaptors) (Glass, *et al.* 1997; Katzenellenbogen, *et al.* 1996; Shibata, *et al.* 1997). Several proteins to date have been associated with gene transcriptional enhancing properties such as SRC-1 (Onate, *et al.* 1995; Spencer, *et al.* 1997), AIB-1 (a member of the SRC-1 family) (Anzick, *et al.* 1997) and RIP140 (Cavailles, *et al.* 1995). Likewise,

transcriptional repressor proteins have been identified (Chen & Evans 1995). Steroid regulated gene expression is further complicated by the finding that some neurotransmitters and growth factors (e.g., epidermal growth factor) can mimic steroid hormone action by a ligand-independent mechanism (Gangolli, *et al.* 1997; Ignar-Trowbridge, *et al.* 1992). Collectively, these studies indicate that steroid-driven gene activation is modulated by multiple factors of which only one component is the receptor. So, although estrogen and progesterone are key hormones in the regulation of breast cancer tumor growth, there are many additional contributory factors (i.e., growth factors and co-factors) that also regulate breast cancer proliferation.

Although steroid hormone receptor levels can be used as a markers to assess extent of tumor progression toward malignancy, few studies directly demonstrate a functional role in this regard, especially with regard to metastasis. The most direct test was by Garcia *et al.* who transfected the ER-negative MDA-MB-231 breast carcinoma cell line with estrogen receptor (ER-α) and then treated the transfectant cells with estrogens and anti-estrogens. Experimental metastatic potential following intravenous inoculation of cells was inhibited 3-fold by estradiol whereas the antiestrogen Tamoxifen had little effect (Garcia, *et al.* 1992). Estradiol also increased the invasive capabilities of these transfectants in an *in vitro* invasion assay using Matrigel; antiestrogens inhibited these effects. Interestingly, in contrast to the typical stimulatory effect of estradiol on ER-positive breast cancer cell growth, estradiol inhibited the cell proliferation of ER-transfectants. These results must be viewed cautiously until further experiments are done to explain this phenomenon or the experiments are replicated in another cell line.

Endocrine regulation does not act independently to regulate breast tumor cell behavior. The biochemical changes resulting from modified ligand and receptor expression and activation, combined with interrelationships with other growth factors and intracellular signaling pathways, reveal a byzantine regulatory machinery. Abnormal tissue growth is due to a disruption of the balance between stimulated proliferation and inhibition of cell death. Transformation and progression can be due to: (1) increased production of growth-promoting factors; (2) decreased synthesis of growth-inhibitory factors; (3) decreased responsiveness to growth-promoting factors; or (4) decreased sensitivity to growth inhibitory signals. The latter two mechanisms can be direct because of alterations in receptors or via modifications in the downstream signaling pathways. For purposes of this review, only selected growth factors will be presented to provide examples as to the complexities of growth regulation of breast cancer growth and progression.

Transforming growth factors

Transforming growth factors (TGFs) were identified initially and named based on their ability to transform selected cell types. This family of growth factors has expanded extensively and is now known to consist of several families of polypeptides (Hartsough & Mulder 1997). These are produced and secreted by normal and cancerous cells. TGF expression can be regulated by steroids as well as by other growth promoting factors, thereby leading to an intricate and complex of negative and positive pathways modulating cell cycle progression or homeostasis. TGF-α and TGF-β represent two distinct families of growth factors that are structurally and functionally distinct.

TGF-α and EGF families

Many members of the TGF-α family compete with epidermal growth factor (EGF) for binding to the EGF receptor. Like EGF, TGF-α binding results in receptor dimerization, activation of tyrosine kinase activity and eventually leads to stimulation of cell proliferation or differentiation (Derynck 1988; Massague 1983; Todaro, *et al.* 1990). Other members of this family include amphiregulin, heparin-binding EGF, cripto-1, and a subfamily of heparin binding proteins called heregulins (the human homolog) (Bates, *et al.* 1988; Higashiyama, *et al.* 1991; Todaro, *et al.* 1990). Heregulin does not appear to bind the classic EGF receptor, but initially was thought to bind instead to a related EGF receptor protein called erbB-2 (HER-2/neu) (Bargmann, *et al.* 1986; Coussens, *et al.* 1985; Schechter, *et al.* 1985; Schechter, *et al.* 1984; Stern, *et al.* 1986; Yamamoto, *et al.* 1986). Studies now indicate that heregulin does not directly bind erbB-2, but rather to two related receptor forms, erbB-3 (Kraus, *et al.* 1989; Plowman, *et al.* 1990) and erbB-4 (Carraway, *et al.* 1994; Plowman, *et al.* 1993). All four receptor forms (EGF receptor, erbB-2, -3 and -4) have been reported present in human breast cancers. In about 30% of human breast cancers, erbB-2 is amplified or overexpressed; this is associated with poor patient prognosis and maintaining the malignant phenotype (Allred, *et al.* 1992; Slamon, *et al.* 1987). Overexpression of HER-2/neu and its relationship with other prognostic factors change during the progression of *in situ* to invasive breast cancers

Overexpression of erbB-2/HER-2/neu and its relationship with other prognostic factors change during the progression of *in situ* to invasive breast cancer (Allred, *et al.* 1992; De Potter, *et al.* 1990; Gusterson, *et al.* 1992; Paik, *et al.* 1990; Toikkanen, *et al.* 1992; Van de Vijver, *et al.* 1988). Because of this, erbB-2 overexpression was thought to be a key factor that increased the invasive potential of breast

cancer cells; however, studies examining comedo-type intraductal carcinomas showed that a higher proportion overexpressed erbB-2 protein compared to invasive cancer, thereby indicating that though erbB-2 overexpression may play a role in invasion, it does not singly lead to increased invasiveness (Van de Vijver, *et al.* 1988). The roles of erbB-3 and -4 in breast cancer invasion and metastasis are not known.

TGF-β family

The TGF-β family of polypeptide growth factors is comprised of several related gene products that form either homodimers or heterodimers. TGF-β isoforms are found in both normal mammary epithelium and in breast tumors. The interactions of these various isoforms is further complicated by the presence of specific binding proteins (Butzow, *et al.* 1993; Chefietz, *et al.* 1988; Murphy-Ullrich, *et al.* 1992; Wakefield, *et al.* 1992). In addition, two TGF-β receptors (type I and type II) have been identified. Four type I receptors have been cloned (Wang, *et al.* 1994). Type I and type II receptors can heterodimerize. Because there are a wide variety of receptor combinations as well as the existence of multiple TGF-β forms, a diverse number of pathways appear available to regulate breast cancer growth and differentiation.

Most normal epithelial cells are growth inhibited when exposed to TGF- β (Arteaga, *et al.* 1996). Restoration of TGF-β receptors in nonresponsive MCF7 cells renders the cells less tumorigenic and proliferative when grown in the presence of TGF-β (Sun L., *et al.* 1994). Because of this, studies on the role of TGF-β in cancer biology have mostly focused on this factor's effect on growth regulation and tumor formation. However, there is accumulating evidence that TGF-β plays a critical role in tumor invasion and metastasis.

TGF-β overexpression in breast tumors has been associated with a more malignant phenotype (Dickson & Lippman 1996). A specific role in invasion and metastasis was demonstrated when Welch and colleagues first showed that exposure of mammary adenocarcinoma cell lines to picomolar concentrations of TGF-β1 or TGF-β2 induced production of metalloproteinases with a corresponding increase in invasiveness and experimental metastatic potential (Welch, *et al.* 1990). At these concentrations, growth inhibition was not observed. Similar findings have been reported for the metalloproteinases as well as the urokinases (Agarwal, *et al.* 1994; Dong-Le, *et al.* 1998; Reiss & Barcellos-Hoff 1997; Sehgal, *et al.* 1996; Walker & Dearing 1992; Walker, *et al.* 1994). It is important to note that the source of the TGF-β can be the tumor cells themselves or nearby host cells. Indeed TGF-β

can increase stromal cell secretion of urokinase (Hildenbrand, *et al.* 1998). Thus, tumor cells which produce TGF-β could manipulate stromal cells to assist in their malignancy. This concept is substantiated by the known roles of TGF-β in angiogenesis and immunosuppression (De Jong, *et al.* 1998a; De Jong, *et al.* 1998b; Enenstein, *et al.* 1992; Relf, *et al.* 1997).

Interestingly, TGF-β expression was originally correlated with increased bone colonization by Walker 256 carcinosarcoma cells (Orr, *et al.* 1993). Since bone is the most common site for breast cancer metastasis, organotropism may be partly explained by differential expression of TGF-β. This hypothesis is at least partially supported by Guise and colleagues who showed that TGF-β can alter expression of parathyroid hormone-related protein (PTHrP) which is, in turn, involved in bone resorption. Expression of PTHrP ± exposure to TGF-β regulates bone colonization by MDA-MB-231 cells (Guise 1997). Still, it must be emphasized that a role for TGF-β in bone colonization by breast cancer has still not been definitively established.

Other growth factors

In addition to the EGF and TGF-β families, numerous other growth factor families have been identified and found in breast cancer cells. These include the insulin-like growth factors (IGF-1 and IGF-2), fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), and vascular endothelial growth factor (VEGF) (Ferrara, *et al.* 1992; Goustin, *et al.* 1986; Heldin & Westermark 1984; Sporn & Roberts 1986). The expression of many of these growth factors can be regulated by estrogen and progesterone (Dickson & Lippman 1996).

Thrombospondin is a 450 kDa adhesive glycoprotein present in high concentrations in the platelet alpha-granule. It is also synthesized by other cells and is incorporated into extracellular matrices. The role of thrombospondin in breast cancer biology is checkered (Qian & Tuszynski 1996; Roberts 1996; Volpert, *et al.* 1995; Walz 1992). Transfection experiments suggest that it can promote cell adhesion, invasion and/or metastasis in some tumor models (Arnoletti, *et al.* 1995; Incardona, *et al.* 1995; Pratt, *et al.* 1989; Tuszynski, *et al.* 1987a; Walz 1992; Wang, *et al.* 1996); whereas, it is suppressive in others (Qian & Tuszynski 1996; Weinstat-Saslow, *et al.* 1994b; Zabrenetzky, *et al.* 1994). Metastasis-promoting effects are often attributed to changes in adhesion whereas, the suppressive effects can be, at least partially, explained by the anti-angiogenic effect of thrombospondin (Dameron, *et al.* 1994a; Dameron, *et al.* 1994b; Volpert, *et al.* 1995; Weinstat-Saslow, *et al.* 1994b). Interestingly, thrombospondin expression is regulated by progesterone in the endometrium (Iruela-Arispe, *et al.* 1996), opening the possibility that

analogous regulation could occur in breast. Also, TSP-1 expression appears to be regulated by p53 (Dameron, *et al.* 1994b), which itself has been implicated in breast tumorigenesis (See TP53 in Table 1).

Thus, there are a multitude of interrelated growth factors, receptor types, and steroid hormones in the normal mammary epithelium that tightly regulate and coordinate cell proliferation and differentiation. In breast cancer cells, the intricate balance is perturbed. Invasive and metastatic cells further circumvent the regulation by overexpression or downregulation of growth factors and/or their receptors. Aberrations of downstream signaling cascades further contribute to cellular delinquency. Delineation of these pathways and their impact on angiogenesis, immune response, growth, invasion, and metastasis will require new models.

Immune regulation of breast cancer metastasis

There is clearly evidence that breast cancer metastasis is based upon the inherent genetic makeup of the tumor cells. However, tumor cells do not exist in isolation and their biological properties are not fully self-determined. Examples are described above. But there is one more that merits mentioning. The role of the immune system in cancer is usually considered to be elimination of tumor cells. But because metastatic cells and activated leukocytes share many properties, including the ability to attach to endothelium (Hoover & Ketcham 1975; Yong & Linch 1993) as well as degradation of and penetration of basement membranes (Wright & Gallin 1979; Klotz & Jesaitis 1994), it was suggested that, under certain conditions, tumor cells might exploit normal leukocyte function to increase metastatic efficiency (Gorelik, *et al.* 1982; Aeed, *et al.* 1988).

Rats injected with syngeneic 13762NF mammary adenocarcinoma cell clones developed neutrophilia proportional to the metastatic potential of the primary tumor (Aeed, *et al.* 1988). We showed that the metastatic tumor variants did so by secreting granulocyte-macrophage colony-stimulating factor (GM-CSF) and/or interleukin-3 (IL-3) in proportion to their metastatic propensity (McGary, *et al.* 1995). More importantly, tumor-elicited neutrophils increased metastatic potential and invasiveness 2- to 25-fold when co-injected intravenously (Welch, *et al.* 1989); whereas, normal circulating neutrophils, proteose peptone-elicited and phorbol ester-activated neutrophils did not. Alone, these findings may have been merely an experimental curiosity. However, anecdotal clinical data suggest that these types of observations are not altogether uncommon. Leukocytosis (Sawyers, *et al.* 1992), granulocytosis (Suda, *et al.* 1980; Hughes & Higley 1952), eosinophilia (Sawyers, *et al.* 1992) and neutrophilia (Lee, *et al.* 1987)

have been described in patients with advanced neoplasms of multiple histologic types. This could not be explained by infection or tumor necrosis (Aeed, *et al.* 1988). In experimental models, the evidence predominantly supports secretion of factors that stimulate bone marrow precursor cells. Lee and colleagues have shown that GM-CSF levels may be correlated with more advanced mammary tumors, (Lee & Baylink 1983; Lee, *et al.* 1987; Lee & Lottsfeldt 1984). Factor(s) produced by other tumor cell types that elicit bone marrow proliferation vary by tumor type, stage and size (Asano, *et al.* 1977; Fu, *et al.* 1991; Mano, *et al.* 1987; Nitta, *et al.* 1992; Sawyers, *et al.* 1992; Wu, *et al.* 1979). Takeda et al. found that 7/14 metastatic transplantable tumors produced GM-CSF mRNA and/or detectable GM-CSF activity; whereas, the nonmetastatic tumors did not (Takeda, *et al.* 1991). Taken together, these results demonstrate that breast cancers may modulate their metastatic potential, in part, by manipulation of the immune system.

A molecular genetic model for breast tumor progression

The collection of neoplastic breast diseases are sufficiently distinct that it is unlikely that a single model could describe the genetic changes leading to metastasis. At the root of any model must be a clear understanding of the cell type from which a particular neoplasm developed. Notwithstanding, the majority of evidence suggests that cells from the terminal ductal structures are the cells of origin. Insufficient biochemical and molecular markers allow for more refinement than that with regard to cellular origin. It is believed that the conversion to neoplasia has an intermediary atypical hyperplasia in which the cells have lost some aspects of growth control, but still retain vestigial response to growth controlling signals. During the proliferative phase, cells are responding to the usual milieu of positive and negative endocrine, paracrine and juxtacrine signals. During this hyperproliferative phase, breast epithelial cells accumulate mutations in oncogenes and tumor suppressor genes so that they appear even less "normal" or differentiated and are classified as carcinomas *in situ*. Further proliferation results in accumulation of mutations, increasing malignant characteristics (i.e., invasion, aneuploidy, angiogenesis, etc.) so that eventually, a subset of cells is no longer confined to the breast.

Over 150 genes and genetic loci have been associated with breast cancer development. Of those changes, this review summarizes evidence implicating a role in progression to malignancy for over forty different genes. The magnitude of these numbers highlight the tremendous complexity of breast cancer as a family of diseases. The good news is that all of these markers have been identified in spite of the extraordinary heterogeneity that exists within breast neoplasms at diagnosis. The bad news is that these

changes are only the tip of the iceberg. How, then, can one determine which changes are essential and which are ancillary?

For oncogenes and tumor suppressor genes, the data in breast cancer oncogenesis is relatively mature. While there is still plenty of room for further study, correlative data are often corroborated by functional studies (i.e., transfection with wild-type cDNA followed by bioassay). Mechanism of action is not always known; however, the biological endpoints are unambiguous. The situation is less clear with regard to genes/loci involved in breast tumor progression, invasion and/or metastasis. Only four genes (Nm23-H1, KiSS-1, KAI1 and TSP-1) have been demonstrated to suppress metastasis of human breast carcinoma cells following orthotopic implantation of tumor cells into immunocompromised mice. Of those, only one, NME1 has been studied adequately in the clinical arena to warrant serious consideration as having prognostic value. KAI1 suppressed metastasis at a level comparable to Nm23, but KiSS-1 was more potent than any of the other genes tested with regard to reduction in metastasis incidence burden. To claim TSP-1 as a metastasis-suppressor gene may be a misnomer since tumor growth was also inhibited. Nonetheless, the tumor cells still expressed the transgene, allowing TSP-1 to still qualify by the criteria listed above.

Considering the number of papers claiming to study metastasis of breast cancer, the number of *bona fide* functionally-tested metastasis-suppressor genes is surprisingly small. In part, this is due to the paucity of models which allow testing *in vivo*. Indeed most of the functional studies were done using the MDA-MB-435 model. Validation in other models has not been done. Certainly, testing in other breast tumor types has not been attempted. Thus, for the breast cancer metastasis field to advance further, more and better models will be required.

Despite the discovery of and identification of four (and probably more) metastasis-suppressor genes, several questions remain regarding control of the metastatic phenotype in human breast cancer. Do the identified genes represent rate-limiting steps? Are these genes functioning in a single pathway or convergent pathways of metastasis control? What are the signals that control these genes? Are the key controlling signals among the correlations already established for breast cancer progression (i.e., hormonal or growth factor control)? While much has been learned, more still remains to be found.

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Table 1. Genes reportedly involved in oncogenesis and/or progression of human breast cancer

Gene	Map Location	Presumptive mechanism(s) of action	Identified Aberrations in Clinical Samples or Cell Cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis [a]
APC / FAP	5q21	Regulate β-catenin; cytoskeletal organization	LOH	(Thompson, *et al.* 1993)	
ATM ataxia-telangiectasia	11q22-q23	DNA repair	LOH, mutation	(Athma, *et al.* 1996; Carter, *et al.* 1994; Cortessis, *et al.* 1993; Ferti-Passantonopoulou, *et al.* 1991; Hampton, *et al.* 1994; Kerangueven, *et al.* 1997; Tomlinson, *et al.* 1995; Vorechovsky, *et al.* 1996)	
α-catenin	5q31	Cytoplasmic component of E-cadherin; cytoskeletal organization	**Reduced expression:** (Glukhova, *et al.* 1995; Rimm, *et al.* 1995) **Mutation:** (Rimm, *et al.* 1995)		**Invasion** (Glukhova, *et al.* 1995; Rimm, *et al.* 1995)
bcl-2	18q21	Apoptosis; interacts with c-myc	Overexpression, amplification		**Progression:** (Olopade, *et al.* 1997; Silvestrini, *et al.* 1994; Zschiesche, *et al.* 1997)
BrCa1	17q21		LOH, mutation	(Casey 1997; Dickson & Lippman 1995; Holt, *et al.* 1996; Rao, *et al.* 1996)	
		DNA repair, Genome stability		(Scully, *et al.* 1997)	
		Cell cycle		(Chen, *et al.* 1996; Futreal, *et al.* 1994; Larson, *et al.* 1997; Miki, *et al.* 1994; Somasundaram, *et al.* 1997; Wang, *et al.* 1997)	

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		Differentiation		(Boyd, *et al.* 1995; Goldman, *et al.* 1997; Hakem, *et al.* 1996; Ludwig, *et al.* 1997; Marquis, *et al.* 1995)	
		Apoptosis		(Shao, *et al.* 1996)	
BrCa2	13q12-q13		LOH, mutation	(Casey 1997; Cleton-Jansen, *et al.* 1995; Collins, *et al.* 1995; Wooster, *et al.* 1995)	
		DNA repair, Genome stability		(Patel, *et al.* 1998; Sharan, *et al.* 1997)	
		Differentiation		(Ludwig, *et al.* 1997)	
		Cell cycle		(Wang, *et al.* 1997)	
BrCa3	8p12-p22	DNA repair	LOH	(Casey 1997; Hoekstra 1997; Lavin & Shiloh 1997; Meyn 1995; Seitz, *et al.* 1997)	
Brush-1	13q12-q13			(Schott, *et al.* 1994)	
Cathepsin D	11p15-pter	Proteinase	Overexpression	(Westley & May 1996)	**Progression/Invasion**: (Garcia, *et al.* 1996; Johnson, *et al.* 1993; Lah, *et al.* 1995; Rochefort, *et al.* 1990a; Rochefort, *et al.* 1990b; Tedone, *et al.* 1997)
CD31 (PECAM)	17q23	Angiogenesis (marker)	Increased expression in stromal component		**Progression/Angiogenesis/Invasion**: (Charpin, *et al.* 1995; Fox, *et al.* 1997; Martin, *et al.* 1997)

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				oncogenesis	clinical stage/grade, tumor progression or metastasis [a]
CD44	11p13	Adhesion	Amplification, Overexpression		**Progression/Invasion/Metastasis**: (Herrlich, *et al.* 1993; Zöller & Kaufmann 1994) **Progression/Invasion/Metastasis**: (Hofmann, *et al.* 1991; Joensuu, *et al.* 1993)
c-erb-B2	17q12	Growth factor receptor Tyrosine kinase	**Amplification:** (28-35%) (Slamon, *et al.* 1987; Spandidos, *et al.* 1989; Zhou, *et al.* 1989) **Overexpression:** (Anbazhagan, *et al.* 1991; Gullick, *et al.* 1991; Lovekin, *et al.* 1991; O'Reilly, *et al.* 1991)	Conflicting/controversial data. Studies are at odds, no consistent or defining studies.	**Stage/Progression**:(Hubbard, *et al.* 1994; Liu, *et al.* 1992; Press, *et al.* 1994; Slamon, *et al.* 1987; Slamon, *et al.* 1989) **Control of metastasis or invasion**: (Giunciuglio, *et al.* 1995; Tan, *et al.* 1997; Tavassoli, *et al.* 1989; Yu & Hamada 1992; Yusa, *et al.* 1990)
c-myc	8q24	**Transcription:** (Bonilla, *et al.* 1988; Edwards, *et al.* 1988) **Growth, Differentiation**: (Evan, *et al.* 1992) **Apoptosis:** (Chernova, *et al.* 1998; Packham, *et al.* 1996; Ryan & Birnie 1996; Wagner, *et al.* 1994)	Amplification, Overexpression, Mutation	(Berns, *et al.* 1992a; Berns, *et al.* 1992b; Dickson & Lippman 1995; Escot, *et al.* 1986; Guerin, *et al.* 1988; Kozbor & Croce 1984; Nass & Dickson 1997; Watson, *et al.* 1996; Wong & Murphy 1991)	**Progression**: (Guerin, *et al.* 1988; Tavassoli, *et al.* 1989; Watson, *et al.* 1993; Watson, *et al.* 1996)

Table 1. Genes reportedly involved in oncogenesis and/or progression of human breast cancer

Gene	Map Location	Presumptive mechanism(s) of action	Identified Aberrations in Clinical Samples or Cell Cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis [a]
Cyclin D1	11q13	Cell cycle	Amplification, overexpression, Mutation	**Amplification**: (Buckley, *et al.* 1993; Courjal, *et al.* 1996; Dickson, *et al.* 1995; Gillett, *et al.* 1994; Han, *et al.* 1995; Peters 1994; Peters, *et al.* 1995) **Mutated**: (Lebwohl, *et al.* 1994) Overexpression: (Bartkova, *et al.* 1994; Bartkova, *et al.* 1995) **In precursor lesion (DCIS to infiltrating ductal Ca)**: (Steeg, *et al.* 1996; Weinstat-Saslow, *et al.* 1995)	
Cyclin E	ND	Cell cycle	Overexpression	(Bortner & Rosenberg 1997; Gray-Bablin, *et al.* 1996)	**Progression:** (Keyomarsi, *et al.* 1994; Said & Medina 1995)
DCC	18q21		LOH	(Thompson, *et al.* 1993)	
E-cadherin	16q22.1	Adhesion - homotypic	**Reduced expression:** (Palacios, *et al.* 1995)	(Hirai, *et al.* 1998; Lochter, *et al.* 1997a)	**Invasion/metastasis:** (Guriec, *et al.* 1996; Jones, *et al.* 1996; Lipponen, *et al.* 1994; Mbalaviele, *et al.* 1996; Oka, *et al.* 1993; Palacios, *et al.* 1995; Siitonen, *et al.* 1996; Berx, *et al.* 1995; Perl, *et al.* 1998; Rimm, *et al.* 1995)
ERα	6q24-q27	Hormone receptor, Transcription	Mutation, Loss of expression LOH	(Andersen, *et al.* 1994)	**Tumor progression:** (Estes, *et al.* 1987; Graham, *et al.* 1990; Leygue, *et al.* 1996b; Mackay, *et al.* 1988; Magdelénat, *et al.* 1994; Scott, *et al.* 1991; Sheikh, *et al.* 1994; Thompson, *et al.* 1992) **Invasion**: (Garcia, *et al.* 1992; Hoelting, *et al.* 1995; Sheikh, *et al.* 1994) **Metastasis**: (Fuqua, *et al.* 1991a; Garcia, *et al.* 1992)

Table 1. Genes reportedly involved in oncogenesis and/or progression of human breast cancer

Gene	Map Location	Presumptive mechanism(s) of action	Identified Aberrations in Clinical Samples or Cell Cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis [a]
ERβ	14q22-24	Hormone receptor, Transcription	Mutation	(Dotzlaw, *et al.* 1997; Enmark, *et al.* 1997; Kuiper, *et al.* 1996; Leygue, *et al.* 1996a; Vladusic, *et al.* 1998)	
ETS-2		Transcription	Overexpression		**Invasion:** (Sapi, *et al.* 1998)
FGF family	multiple	Growth factors, Angiogenesis	Amplification, Overexpression	(McLeskey, *et al.* 1996; Payson, *et al.* 1996; Penault-Llorca, *et al.* 1995; Relf, *et al.* 1997)	**Progression:** (Souttou, *et al.* 1996) **Metastasis**: (Kern, *et al.* 1994; McLeskey, *et al.* 1993)
FHIT	3p14.2		LOH Mutation	(Mau, *et al.* 1996; Negrini, *et al.* 1996; Panagopoulos, *et al.* 1996)	
		Fragile histidine triad; Genomic stabilty		(Barnes, *et al.* 1996; Huebner, *et al.* 1997)	
		Hormone receptor, Transcription		(Martin, *et al.* 1993; Tonetti & Jordan 1997)	
IGF2R (mannose 6-phosphate receptor)	6q26-q27		Overexpression		**Progression**: (Chappell, *et al.* 1997)
IL-1β	2q13	Cytokine	Increased expression		**Progression:** (Jin, *et al.* 1997)
IL-8	4q13	Cytokine	Increased expression		**Progression/Angiogenesis**: (Green, *et al.* 1997)
int-1	12q13		Amplification	(Meyers, *et al.* 1990)	
int-2/FGF-3	11q13	Growth factor	Amplification Overexpression	(Huebner, *et al.* 1988; Liscia, *et al.* 1989)	

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Gene	Map Location	Presumptive mechanism(s) of action	Identified Aberrations in Clinical Samples or Cell Cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis [a]
KAI-1 (CD82)	11p11.2	Adhesion	Decreased expression		**Progression:**(Yang, *et al.* 1997) **Transfection/Metastasis:**(Phillips, *et al.* 1998)
KiSS-1	1q32	Signal transduction	Decreased expression		**Transfection/Metastasis**: (Lee & Welch 1997c)
Laminin-5	1q	Adhesion, Invasion	Overexpression		**Invasion**: (Pyke, *et al.* 1995)
mdm-2	12q13-q14	Inhibit TP53	Overexpression	(Jiang, *et al.* 1997)	**Progression:** (Jiang, *et al.* 1997)
MMPs / TIMPs	multiple	Invasion		(Lochter, *et al.* 1997a; Lochter, *et al.* 1997b)	**Progression:** (Tryggvason, *et al.* 1993) **Experimental Models**: (Polette, *et al.* 1997; Stonelake, *et al.* 1997; Ueno, *et al.* 1997; Wang, *et al.* 1997)
		Angiogenesis		(Thorgeirsson, *et al.* 1996)	
MnSOD (SOD2)	6q25	Reduce oxygen radicals	Decreased expression	(Li, *et al.* 1995)	
MRP-1/CD9	12p13	Differentiation; Motility	Loss of expression		**Progression**: (Miyake, *et al.* 1995; Miyake, *et al.* 1996)
NFκB		Transcription	Overexpression	(Sovak, *et al.* 1997)	

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Gene	Map Location	Presumptive mechanism(s) of action	Identified Aberrations in Clinical Samples or Cell Cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis [a]
NME1 Nm23-H1	17q21.3	NDP kinase? Some find that NDPK activity is not associated with metastasis suppression (MacDonald, *et al.* 1993)	Decreased expression, Mutation		**Lymph node status:** (Barnes, *et al.* 1991; Bevilacqua, *et al.* 1989; Freije, *et al.* 1996; Hennessy, *et al.* 1991; Royds, *et al.* 1993; Steeg, *et al.* 1993; Tokunaga, *et al.* 1993; Toulas, *et al.* 1996) **Histologic grade:** (Hirayama, *et al.* 1991; Yamashita, *et al.* 1993) **No correlation:** (Goodall, *et al.* 1994; Sastre-Garau, *et al.* 1992; Sawan, *et al.* 1994) **Transfection/Metastasis:** (Fukuda, *et al.* 1996; Leone, *et al.* 1993)
		Growth		(Cipollini, *et al.* 1997)	
NME2 Nm23-H2	17q	NDP kinase			**Transfection/Metastasis:** (Fukuda, *et al.* 1996; Kraeft, *et al.* 1996)
		c-myc transcription			**Transfection:** (Postel, *et al.* 1993) **No suppression:** (Tokunaga, *et al.* 1993)
Nm23-DR	ND	Differentiation, Apoptosis			
Nm23-H4	16p13	NDP kinase			
p16/p15/p19^{ARF}	9p21	Cell cycle	Mutation, LOH (Haber 1997)	(Brenner & Aldaz 1995; Geradts & Wilson 1996; Herman, *et al.* 1995; Xu, *et al.* 1994; Zariwala, *et al.* 1996)	
p21$^{WAF1/CIP1/mda6/sdi1}$	6p21	Cell cycle	**Overexpression:** (Lukas, *et al.* 1997) **Decreased expression:**(Jiang, *et al.* 1997)	(Lukas, *et al.* 1997; Rey, *et al.* 1998)	**Progression:** (Jiang, *et al.* 1997)

Table 1. Genes reportedly involved in oncogenesis and/or progression of human breast cancer

Gene	Map Location	Presumptive mechanism(s) of action	Identified Aberrations in Clinical Samples or Cell Cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis [a]
p53 (TP53)	17p13.1		LOH, Mutation, Mutant overexpression (Bennett, *et al.* 1992; Gusterson, *et al.* 1991)	(Bartek, *et al.* 1990; Bukholm, *et al.* 1997; Davidoff, *et al.* 1991; Eyfjörd, *et al.* 1995; Gusterson, *et al.* 1991; Harris 1992; Hartmann, *et al.* 1997; Horak, *et al.* 1991; Jerry, *et al.* 1993; Poller, *et al.* 1992)	**Progression:** (Allred, *et al.* 1993; Anbazhagan, *et al.* 1991; Barnes, *et al.* 1993; Casey, *et al.* 1993; Chen, *et al.* 1994; Gullick, *et al.* 1991; Lovekin, *et al.* 1991; Mazars, *et al.* 1992; O'Reilly, *et al.* 1991; Poller, *et al.* 1992; Thor, *et al.* 1992)
		Transcription		(Harris 1996; Levine 1997; Wang & Harris 1997)	
		Genome stability		(Levine 1997; Tlsty, *et al.* 1993; Wynford-Thomas 1997)	
PR	11q13	Hormone receptor, Transcription; marker for estrogen response	Decreased expression, Mutation, LOH		**Progression:** (Ali, *et al.* 1987; Fuqua, *et al.* 1991b; Horwitz, *et al.* 1982; Magdelénat, *et al.* 1994; McGuire, *et al.* 1986; Tomlinson, *et al.* 1996)
PKCα	17q22-q23.2	Signal transduction			**Invasion/Metastasis:** (Ways, *et al.* 1995)
PKCδ		Signal transduction	Overexpression Activation	(Jaken, *et al.* 1997; Kiley, *et al.* 1996)	**Transfection/Metastasis:** (Jaken, *et al.* 1997; Kiley, *et al.* 1996; Kiley, *et al.* 1998)
Mammaglobin	11q13	Steroid binding?	Overexpression, Amplification	(Watson & Fleming 1996)	
MMAC1/PTEN	10q23	Tyrosine phosphatase	LOH, mutation, Decreased expression	**Low importance:** (Chen, *et al.* 1998)	**Progression:** (Dahia, *et al.* 1997; Li, *et al.* 1997; Liaw, *et al.* 1997; Lynch, *et al.* 1997; Nelen, *et al.* 1997; Okami, *et al.* 1998; Rasheed, *et al.* 1997; Rhei, *et al.* 1997; Sakurada, *et al.* 1997; Steck, *et al.* 1997; Teng, *et al.* 1997)

Table 1. Genes reportedly involved in oncogenesis and/or progression of human breast cancer

Gene	Map Location	Presumptive mechanism(s) of action	Identified Aberrations in Clinical Samples or Cell Cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis [a]
Ras	11p15	Signal transduction	**Overexpression:** (Spandidos, *et al.* 1989; Thor, *et al.* 1986) **Mutations but rare**: (Rochlitz, *et al.* 1989; Thor, *et al.* 1986) **LOH**: (Theillet, *et al.* 1986)	(Thor, *et al.* 1986)	**Invasion** (data controversial and contradictory): (Lundy, *et al.* 1986; Spandidos, *et al.* 1989)
Raf-1		Signal transduction	Overexpression (measured in cell lines only)		**Progression**: (Callans, *et al.* 1995)
Rb1	13q14	Cell cycle	LOH, mutation	(Picksley & Lane 1994; Riley, *et al.* 1994; Sherr 1994; Wang, *et al.* 1994) (Cox, *et al.* 1994; Lundberg, *et al.* 1987; Shackney & Shankey 1997; Spandidos, *et al.* 1989; T'Ang, *et al.* 1988; Zhou, *et al.* 1989)	**Progression**: (Borg, *et al.* 1992a; Varley, *et al.* 1989)
Telomerase		Maintain telomere length	Increased activity		**Progression**: {Hoos, Hepp, et al. 1998 ID: 11039}
TSP-1	15q15-q21		LOH, mutation, decreased expression, truncation	(Weinstat-Saslow, *et al.* 1994b; Zabrenetzky, *et al.* 1994; Zajchowski, *et al.* 1990)	**Progression**: (Walz 1992)
		Inhibit angiogenesis		(Castle, *et al.* 1997; Dameron, *et al.* 1994a; Dameron, *et al.* 1994b; Volpert, *et al.* 1995; Weinstat-Saslow, *et al.* 1994b)	**Transfection/Metastasis**: (Weinstat-Saslow, *et al.* 1994b)
		Induce apoptosis		(Guo, *et al.* 1997)	

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Gene	Map Location	Presumptive mechanism(s) of action	Identified Aberrations in Clinical Samples or Cell Cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis [a]
			Increased expression		**Pro-Invasion**: (Albo, *et al.* 1997; Arnoletti, *et al.* 1995; Tuszynski, *et al.* 1987a; Wang, *et al.* 1996) **Pro-Adhesion**: (Incardona, *et al.* 1995; Pratt, *et al.* 1989; Tuszynski, *et al.* 1987b) **Anti-metastatic**: (Zabrenetzky, *et al.* 1994) **Conflicting data (no correlation)**: (Bertin, *et al.* 1997)
TGF-α	2p11-p13	growth factor; synergistically induces mammary tumors with c-myc transgenic animals	Increased expression	**Experimental systems**: {Amundadottir, Nass, et al. 1996 ID: 10689}	
TGF-β1	19q	growth factor; can promote VEGF, or MMP expression	Increased protein expression, Mutation	(Park, *et al.* 1997) **Growth inhibitor:** (Arteaga, *et al.* 1996; Butta, *et al.* 1992; Mazars, *et al.* 1995; Sun L., *et al.* 1994)	(Note: conflicting data that TGF-β1 inhibits or promotes progression) **Increased invasiveness**: (Hildenbrand, *et al.* 1998; Oft, *et al.* 1996; Welch, *et al.* 1989) **Progression**: (Cardillo, *et al.* 1997) **Possible role in metastasis**: (Walker, *et al.* 1994)
TIMP-1	Xp11.23-p11.4	Inhibitor of MMPs	Increased expression	(Li, *et al.* 1994)	**Progression/Invasion:** (Yoshiji, *et al.* 1996b)
TIMP-2	17q	Inhibitor of MMPs	Increased expression		**Progression/Invasion**: (Visscher, *et al.* 1994)

Table 1. Genes reportedly involved in oncogenesis and/or progression of human breast cancer

Gene	Map Location	Presumptive mechanism(s) of action	Identified Aberrations in Clinical Samples or Cell Cultures	References containing or citing evidence for roles in:	
				oncogenesis	clinical stage/grade, tumor progression or metastasis [a]
uPA / tPA PAI-1 / PAI-2	various	Invasion	Increased expression (proteinases) Decreased expression (inhibitors)		**Progression**: (Duffy, *et al.* 1996; Foekens, *et al.* 1995; Ishikawa, *et al.* 1996; Sappino, *et al.* 1987)
VEGF	6p12-p21.3	Angiogenesis	Overexpression		**Progression**: (Anan, *et al.* 1996; Guidi, *et al.* 1997; Kern & Lippman 1996; Yoshiji, *et al.* 1996a; Yoshiji, *et al.* 1997)
VHL	3p25-p26	Cell cycle; inhibits VEGR mRNA accumulation; binds to elongin	mutations	(Beroud, *et al.* 1998)	
WNT	Wnt14 1 Wnt13 1p13 Wnt15 17q21 Wnt3 17q21 Wnt5a 3p14-p21 Wnt10b 12q13	Most data is in murine tumors, but possible correlations exist in human breast carcinomas.		**Wnt-2** (Dale, *et al.* 1996) **Wnt14 and Wnt15** (Bergstein, *et al.* 1997) **Wnt10b** (Bui, *et al.* 1997)	

[a] Progression indicates only that correlations have been seen in clinical and/or experimental systems corresponding with advanced stage or grade. Attributes of later stages of progression for which specific data are correlated are noted. Table 1 contains some data from other models, particularly with regard to mechanism of action. However, most of the data presented are from breast or mammary tumors.

Table 2: Percentage of breast carcinomas showing chromosomal aberrations.

	Chromosome																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
Primary Tumor																							
Structural (p-arm)	7	-	4	1	1	4	1	2	1	-	4	-	-	-	-	-	-	-	-	-	-	-	-
Structural (q-arm)	2	5	1	2	1	2	4	2	2	1	2	2	1	-	-	2	1	-	1	-	-	-	1
Numerical (gain)	-	-	-	-	1	-	2	-	1	-	1	-	-	-	-	-	-	-	-	1	1	-	-
Numerical (loss)	2	-	-	4	1	2	2	5	5	5	5	2	2	2	2	5	6	2	5	-	2	2	2
Metastases																							
Structural (p-arm)	22	-	18	6	10	8	8	8	10	-	8	10	4	-	2	-	2	-	-	-	-	-	2
Structural (q-arm)	20	12	10	4	4	12	16	4	6	2	14	10	4	2	2	6	2	-	-	-	4	2	-
Numerical (gain)	-	4	6	8	10	2	15	6	4	4	4	6	4	8	4	2	4	10	4	8	10	6	8
Numerical (loss)	10	14	6	6	8	6	4	4	6	8	10	10	8	8	6	8	6	6	6	4	6	10	4

Data presented here are adapted from (Emerson, *et al.* 1993; Hill, *et al.* 1987; Trent, *et al.* 1993) using karyotypic analyses of short-term cultures from recently removed breast tumor tissue (primary tumor or metastases). While the overall values vary by study, the relative involvement is consistent in other studies using comparative genomic hybridization (Devilee & Cornelisse 1994a; Devilee, *et al.* 1994b; Gray, *et al.* 1994; Kallioniemi, *et al.* 1994).

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